THE

BIOCHEMICAL JOURNAL

CAMBRIDGE UNIVERSITY PRESS
LONDON: FETTER LANE, E.C. 4

H. K. LEWIS & CO., 136 GOWER STREET, LONDON, W.C. 1
WHELDON & WESLEY, LTD., 2, 3 AND 4, ARTHUR ST., NEW OXFORD ST., LONDON, W.C. 2
PARIS: LIBRAIRIE HACHETTE & CIE
CHICAGO: THE UNIVERSITY OF CHICAGO PRESS
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THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

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AND

ARTHUR HARDEN

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VOLUME XVIII 1924

CAMBRIDGE
AT THE UNIVERSITY PRESS
1924

PRINTED IN GREAT BRITAIN

Vol. XVIII, No. 1 1924

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CAMBRIDGE UNIVERSITY PRESS
C. F. CLAY, Manager
LONDON: Fetter Lane, E.C. 4
also
H. K. LEWIS & Co., Ltd., 136, Gower Street, London, W.C. 1
WHELDON AND WESLEY, Ltd., 2–4, Arthur Street, New Oxford Street, W.C. 2
CHICAGO: The University of Chicago Press
(Agent for the United States and Canada)
BOMBAY, CALCUTTA, MADRAS: Macmillan and Co., Ltd.
TOKYO: The Maruzen-Kabushiki-Kaisha

1924

Price Twenty Shillings nett

PRINTED IN GREAT BRITAIN

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This Society has been instituted for the purpose of facilitating intercourse between those biologists and chemists who are interested in the investigation of problems common to both, such as the chemical problems connected with Agriculture, Brewing, Animal and Vegetable Physiology and Pathology, &c. Persons interested in Biochemistry are eligible for election.

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I. THE BLOOD VOLUME AND THE TOTAL AMOUNT OF HAEMOGLOBIN IN ANAEMIC RATS.

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(*Received October 5th, 1923.*)

It has been shown [Scott, 1923, 1] that in blood regeneration after a series of haemorrhages rats fed upon white bread and whole milk show a marked contrast to rats on a similar diet with an *ad lib.* amount of green food added, the former developing a sub-chronic anaemia and the latter not. An easier method of producing this anaemia in rats has also been demonstrated [Scott, 1923, 2], that of breeding from mothers which were fed for some time before mating and during gestation and suckling on a diet of bread and milk, and rearing the young on the same diet. By such means it is an easy matter to obtain numbers of young rats which show up to about the twenty-fourth week of life an anaemia in which each cubic millimetre of blood shows a diminution of haemoglobin and a normal or increased corpuscular content, *i.e.* a fall in colour index.

It seemed probable that the deficient haemoglobin content in each mm^3. of blood was due to a deficiency in the material from which the pigment could be formed and it became a matter of interest to investigate whether the slow formation of haemoglobin was reflected in other characters of the blood such as its total volume, the total number of cells or the age of the corpuscle. In respect to the last point it will be clear that even if the haemoglobin was more slowly formed and the colour index was depressed the body might accumulate a normal quantity of haemoglobin were there a lower death-rate and a greater longevity of the corpuscle; in such a condition the whole number of corpuscles in the body would increase as would the volume of the blood. Apart from these considerations it was a matter of great interest in view of the work of Lorrain Smith [1900] on human chlorosis and that of Haldane on ankylostomiasis to determine in these rats what the total amount of haemoglobin in the body was, whether there was, as in the case of Lorrain Smith's subjects, an increase in blood volume sufficient to compensate for the diminution in the haemoglobin percentage and to bring the total amount of haemoglobin in the body

up to the normal proportion per 100 g. body weight, or whether there was no such increase in blood volume so that the total haemoglobin in the body fell *pari passu* with the haemoglobin percentage of the blood. This question was therefore the first to be taken up by us.

Methods.

The method of determining the blood volume was the CO method and in principle was the same as that used by Haldane, Lorrain Smith, Boycott, Douglas and others. Instead, however, of determining the CO percentage of the blood colorimetrically, as they did, we used Hartridge's reversion spectroscope for its determination.

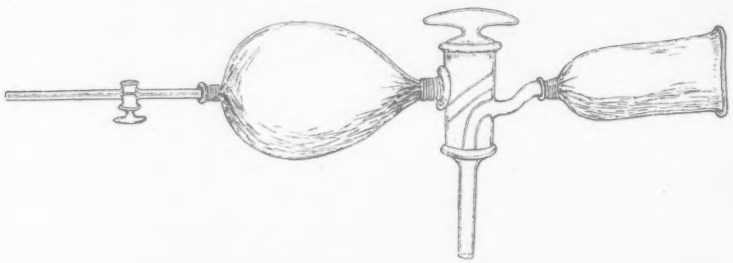


Fig. 1. Apparatus used.

Apparatus (Fig. 1). A mask was made of a size 5 finger-stall which fitted over the head and ears of the rat. In the case of thin finger-stalls a ring cut from a piece of rubber tubing was used as a muzzle; no such muzzle was found necessary in the case of stout finger-stalls. An opening in the tip of the finger-stall connected with a three-way stopper tap on the other limb of which was a balloon of 100 cc. capacity. The bore of the three-way tap and the tubing attached to it were of ample diameter and the tap was disposed so that the rat could breathe either from the open air or the balloon. In an opening at the other end of the balloon was a straight piece of capillary glass tubing with a tap for the introduction of the gas. This apparatus is rather more simple in construction than that used by Boycott and Douglas [1909] in their work on the rabbit. When the apparatus was to be used about 10 g. of soda lime were placed in the balloon and it was wired on to the tap; 15 cc. of oxygen were introduced into it and a carefully measured quantity of CO, usually about 0·5 cc. at N.T.P. One operator holding the rat then thrust its head into the mask, which was held stretched by the other operator and was adjusted by him to cover the head and ears of the rat. The rat was then plunged into a bell-jar of warm water at about 38° up to and covering the free edge of the mask on its neck, so that any leakage of gas might be detected; but care was taken to keep the rat's nose above the level of the water lest water might enter and it should drown. The tap, through which the mask had hitherto communicated with the external air, was turned so that the mask communicated with the

CO mixture in the balloon. The bag was given a little pressure with the hand rhythmically so as to aid the respiration of the rat in mixing the air in the bag with that in the mask, and the soda lime was continually shaken up to absorb CO_2. The duration of administration of CO lasted for times varying between 4 and 10 minutes but was usually between 5 and 8 minutes and was registered by a stop-watch. During this time the legs of the rat were held by the one operator, the body of the rat, the mask and apparatus by the other. The rat usually breathed normally for 2–3 minutes and then commenced to struggle. Breathing usually grew gradually more and more spasmodic and ceased after a variable interval (for what reason we never ascertained), though the heart continued beating. It was an advantage that the circulation should be maintained for some time after the respiration ceased, as the CO taken into the body before the cessation of respiration could be distributed more evenly. In order to prolong the life of some of the control rats we gave them urethane so as to obviate struggling, and this was successful; in a number of such cases we were able to maintain the breathing over a longer period. When the inhalation of CO was concluded, sometimes after respiration had ceased, but always while the heart was still beating, the rat was lifted out on to an enamelled tray; its head was snipped off instantly with a large pair of scissors, and samples of blood were taken for the reversion spectroscope and the haemoglobinometer, care being specially taken in the latter case lest water from the drenched fur should dilute the specimen. Blood was taken in this way so that a satisfactory sample from the central circulation might be obtained and to avoid the lapse of any time in which the body might lose CO. In the rat it is difficult to get from the tail a satisfactory sample of blood without such care as is precluded by the manipulations to which the rat is being subjected. Lorrain Smith's samples were of course taken from the peripheral circulation.

The percentage saturation of the blood with CO was then determined by the reversion spectroscope and the haemoglobin percentage by Haldane's haemoglobinometer. The amount of CO left in the bag was determined with a Haldane's gas analysis apparatus and the amount so left was subtracted from the amount administered. The amount of CO left in the bag was usually trifling and of the order to be expected if the blood and the gas in the bag were in equilibrium. Five quantities required measurement, viz. amount of CO administered, weight of rat, haemoglobin percentage, amount of CO left in the bag, and percentage saturation of the blood with CO. Of these one observer usually measured the first three, the other the last two; in spite of great variations in one or other of these factors the results would fall into line with remarkable consistency. In evolving the routine a number of experiments were performed on about thirty rats.

That the time given is long enough for the absorption of the CO and its equable distribution throughout the circulation is shown by the fact that no difference in blood volume per 100 g. could be detected between rats in which the administration lasted 5 and those in which it lasted 10 minutes. The

"circulation" is here held to exclude the spleen pulp into which the CO scarcely entered. On the other hand, the blood in the liver seemed to be saturated to the same extent as that of the general circulation. The same was true of the maternal placental blood in pregnant females and approximately, though not entirely so, of the blood in the foetal portions of the placenta.

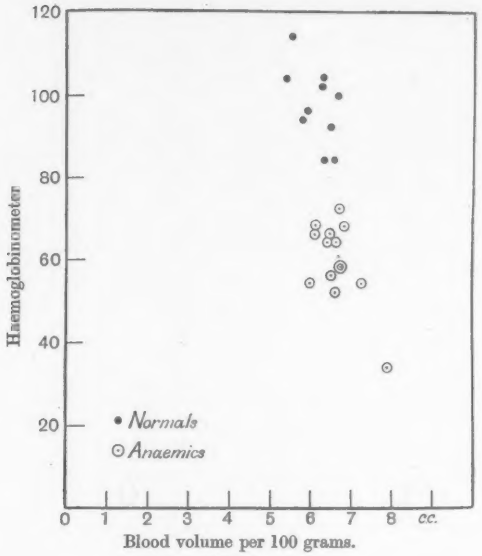
Comparison with results obtained by Welcker's method.

With the co-operation of Messrs Savage and Ellis an attempt was made to compare the results of the CO method of estimating blood volume in rats with those of the Welcker method. The latter method gave invariably smaller results and it was found that this was due to the fact that in the rat haemoglobin shows a great tendency to crystallise. When the blood was washed out of the blood vessels with warm Ringer and diluted by the addition of distilled water to the point at which the solution contains between 1 and 2 % haemoglobin, it still required further laking by alternate freezing and thawing before the solution became clear. Under such circumstances and at such a dilution there was a deposit of haemoglobin crystals in the sediment at the bottom of the receptacle. Thus the method is unsatisfactory for determining blood volume in the rat. The results we did get by the Welcker method were of the same order as those of Kronecker and Marti [1897] who find that the blood volume of a well-nourished rat determined by this method averages 4·7 % of the body weight.

In rabbits Boycott [1912, 1] found that the blood volume given by the CO method was higher than that given by the wash-out method, but in these animals crystallisation of haemoglobin was not observed, and this observer found the explanation in the hypothesis that the CO method, unlike the other, measured extra- as well as intra-vascular haemoglobin.

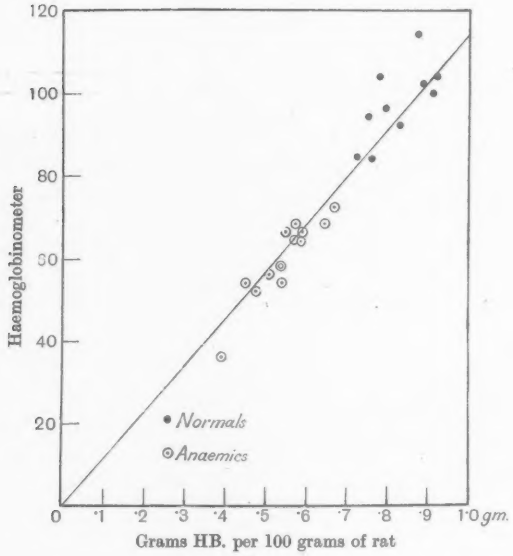
Results.

The normal and anaemic rats compared by us were the same age, viz. 2–3 months. The normals were bred from a normal stock fed with a plentiful supply of green food. The anaemic rats were bred as had been described by Scott [1923, 2]. Both sets were taken at random from large stocks of normal and anaemic rats. The figure we obtain for the average blood volume in normal rats (6·13 cc.) per 100 g. of rat, is very close to that found by Boycott [1912, 2], viz. 6·28 cc. From columns in Tables I (normal) and II (anaemic) rats, the results of which are shown graphically in Fig. 2, it will be seen that there is little difference in blood volume, as determined by the methods described, between the normal and the anaemic rats. The blood volume of the anaemic rats averages 6·65 cc. per 100 g. rat as against 6·13 cc. in the case of the normals. The blood volume in the case of these anaemic rats does not therefore increase appreciably, as would appear from Lorrain Smith's figures (confirmed by Plesch and Oerum) to be the case in human chlorosis. In other columns of Tables I and II are given the figures for the haemoglobin content per 100 g.



Blood volume per 100 grams.

Fig. 2.



Grams HB. per 100 grams of rat

Fig. 3.

Table I. *Blood volume and haemoglobin content per* 100 *g. body weight of* normal *rats about* 2 *months old.*

No.	Weight in g.	Sex	Duration of administration in mins.	CO in cc. Given	Left	Absorbed	% saturation of blood with CO	Hb %	Per 100 g. body weight: Blood volume in cc.	Haemoglobin in g.
49	145	F.	5	·571	·040	·531	34·5	96	5·98	·793
50	140	F.	5½	,,	·037	·534	32·2	102	6·26	·884
51	95	F.	8	,,	·044	·527	47·5	114	5·55	·874
52	96	F.	5	,,	·083	·488	41·5	100	6·64	·916
53	154	M.	6	,,	·027	·544	34·7	84	6·57	·760
54	108	F.	6	·556	·008	·548	41·5	104	6·36	·913
55	134	M.	8	,,	·033	·523	38·7	94	5·80	·754
56	136	M.	6	,,	·030	·526	39·5	84	6·32	·721
57	128	F.	8	,,	·010	·546	41·0	104	5·40	·780
58	106	M.	8	,,	·060	·496	42·5	92	6·47	·822
							Average	97·4	6·13	·822

Table II. *Blood volume and haemoglobin content per* 100 *g. body weight of* anaemic *rats about* 2 *months old.*

No.	Weight in g.	Sex	Duration of administration in mins.	CO in cc. Given	Left	Absorbed	% saturation of blood with CO	Hb %	Per 100 g. body weight: Blood volume in cc.	Haemoglobin in g.
32	118	M.	6	·581	·055	·526	60·0	66	6·09	·555
33	96	M.	7	,,	·054	·527	76·2	58	6·72	·538
34	106	M.	6	,,	·029	·552	68·0	64	6·46	·571
35	127	M.	5½	,,	·045	·536	70·5	54	6·00	·447
36	107	M.	5½	,,	·002	·579	62·7	68	6·85	·643
37	111	F.	6½	,,	·014	·567	64·5	66	6·50	·590
38	98	F.	5	,,	·027	·554	72·5	64	6·57	·582
39	128	M.	5	·577	·064	·513	76·0	36	7·92	·392
40	113	M.	7	,,	·024	·553	72·0	56	6·56	·507
41	100	F.	8	,,	·013	·564	63·2	72	6·70	·666
42	96	F.	5	,,	·066	·511	73·5	54	7·24	·540
43	123	M.	8	,,	·007	·570	60·0	68	6·14	·576
45	88	M.	8	·493	·024	·469	83·9	52	6·60	·474
46	83	M.	10	·492	·055	·437	73·5	58	6·70	·535
							Average	59	6·65	·544

rat and in Fig. 3 is shown the result of plotting this figure against the haemoglobin percentage of the blood. The average haemoglobin percentage in this series of anaemic rats is 59·7 % as against a normal of 97·4 %, and the average total haemoglobin content per 100 g. of rat is 0·544 g. as against a normal of 0·822, a fall to 66 % of the normal. There is therefore no doubt that the animals show a very definite deficiency of haemoglobin. In Fig. 4 are plotted the points representing the averages for normal and anaemic rats respectively. From these charts it will be seen that the total amount of haemoglobin per 100 g. of rat falls in direct proportion to the haemoglobin percentage of the blood, and that this rule holds alike for normal and anaemic rats, so that the points as plotted lie along a straight line the equation of which is

$$y = 115 \begin{pmatrix} +15 \\ -21 \end{pmatrix} x$$

or

$$x = \cdot 0087 \begin{pmatrix} +\cdot 0019 \\ -\cdot 0010 \end{pmatrix} y,$$

in which x = the weight of haemoglobin in grams per 100 g. of rat and y = the haemoglobinometer reading.

Therefore in the case of the rats rendered anaemic by the method described by Scott [1923, 2] it is clearly demonstrated that the total haemoglobin content of the body is not maintained but falls in direct proportion to the haemoglobin percentage of the blood.

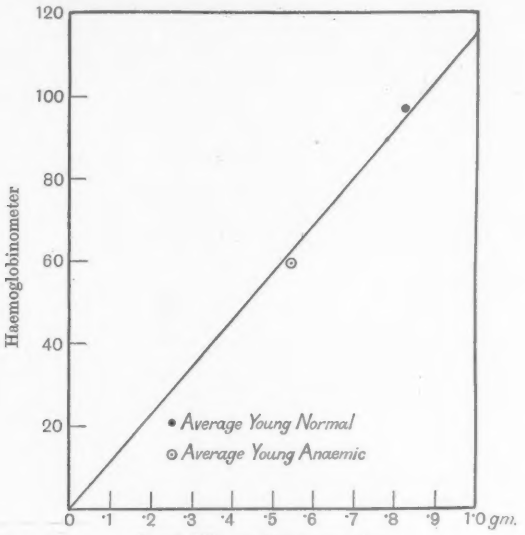


Fig. 4.

Conclusion.

In the anaemia described in rats by Scott [1923, 2] there is a fall in the haemoglobin percentage of the blood, the blood volume per 100 g. remains practically normal, and there is a deficiency in the weight of haemoglobin per 100 g. body weight corresponding and directly proportional to the fall in haemoglobin value.

This relationship is expressed in the equation given in the text.

This rule holds even in the case of such slight diminutions of haemoglobin percentage as occur in normal rats.

Note.

In the course of this work we by chance had the opportunity of noting that pregnant rats resembled anaemic rats in that they showed a slight fall in the haemoglobin percentage of the blood without increase in blood volume, so that per 100 g. weight of rat (including foetuses) they showed a proportional

deficiency in the total haemoglobin content of the body. This is being made the subject of further investigation.

The expenses of this research were in part defrayed by a grant from the Medical Research Council to one of us (J. B.).

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II. FEEDING EXPERIMENTS IN CONNECTION WITH VITAMINS A AND B.

(V) ORANGE JUICE AS A SOURCE OF VITAMIN B.

(VI) OPHTHALMIA IN RATS AFFECTED WITH AVITAMINOSIS.

(VII) THE VITAMIN CONTENT OF COD-LIVER OIL AND MALT EXTRACT.

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(*Received August 13th, 1923.*)

V.

Osborne and Mendel [1920] found that the fresh juice of the orange contained vitamin B in an amount equivalent to that in an equal volume of milk. The experiment to which the present paper relates was carried out with a view to confirming this finding and rats of somewhat mature age were used. Four males and four females, averaging 150 g. in weight and 112 days in age, were fed upon the usual basal dietary with the addition of butter. The amount of the latter was such that each animal received the equivalent of 1·5 g. of butter fat per day. In addition, each animal received 10 cc. of orange juice per day.

The technique of feeding, weighing, etc., was as previously described [Stammers, 1921] and the experiment was carried on for 91 days.

As a control, a comparable set of animals of the same age and average weight was fed upon basal diet plus butter, to which the usual allowance of antineuritic (2 cc. marmite extract) was added. In neither case did the animals receive any lemon juice, which is the usual antiscorbutic supplied, so that the orange juice animals had the advantage over the controls in respect of the antiscorbutic contained in the orange juice.

The average weight of all the animals was about 15 g. subnormal for their age and in the case of the orange juice experiment a progressive decline in weight was observed until the 45th day when the curve of growth began to rise. This rise proved to be only temporary and at the conclusion of the experiment the average weight was only 125 g., the normal for this age being 223 g. These animals had lost 25 g. over the period of the experiment. The

controls also showed a loss of weight at the beginning and this also proved to be temporary, for, after the 14th day they began to gain and continued to do so until the conclusion of the experiment when the average weight was 183 g. They thus gained 33 g. during the course of the experiment.

A graph is appended (Fig. 1) showing the growth recorded in each case and Donaldson's normal curve for the age of the animals under observation.

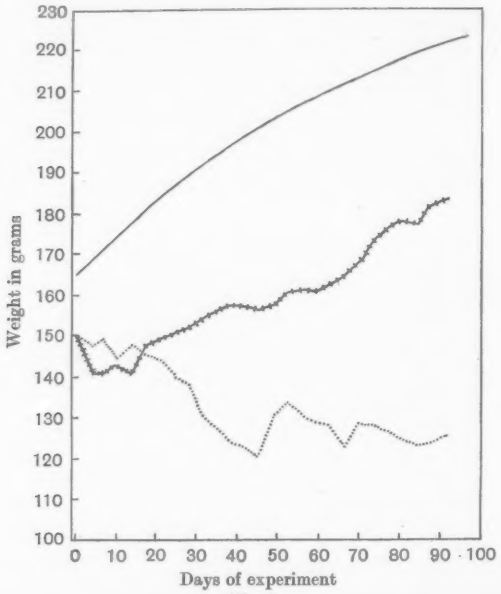

Fig. 1.

——— Normal (Donaldson). ⊣⊣⊣⊣⊣⊣ Butter + Marmite.
• • • • • • Butter + Orange Juice.

Summary and Conclusions.

Eight rats, each supplied with 10 cc. per day of orange juice in addition to the usual basal ration plus butter, failed to maintain their initial weight, whereas eight others given 2 cc. of marmite extract in addition to the basal ration plus butter exhibited growth which, while not normal, yet indicated that the vitamins in the diet supplied were adequate

The source of vitamin A in the diet was the same in both cases so that it appears that the marmite extract in the latter was the factor concerned in the superior growth shown by the control animals. It is pointed out that, whereas the controls got no antiscorbutic, the orange juice animals derived vitamin C from the fruit juice.

Looking upon orange juice, therefore, merely as a source of vitamin B, it appears that 10 cc. of this per day are not sufficient to promote adequate

growth even though there is a sufficiency of vitamin A in the diet. This quantity, however, appears to be protective against the disease associated with deficiency of vitamin B, no symptoms having been noticed during the course of the experiment, and the animals, apart from their loss of weight, appearing healthy.

VI.

Xerophthalmia or keratomalacia has been observed to develop in rats as a result of deprivation of vitamin A. It is not however maintained that this is the sole cause of the disease since it has been noted as a result of unsatisfactory relations in the inorganic portion of the diet [McCollum, Simmonds and Becker, 1922]. It has been well known for some time that a primary symptom of malnutrition among children is an ophthalmia, due to lowered resistance, which offers a focus for bacterial invasion at one of the weakest points in the body.

Histologically, it has been shown that the condition, among rats fed on a diet deficient in vitamin A, results from a degeneration of corneal tissue caused by bacterial invasion, but that this only occurs after a prolonged course of the deficient diet [Stephenson and Clark, 1920]. Yudkin and Lambert [1922] find that the eyelid is primarily affected and this is also the experience of the author. Further, they observe a degeneration of the epidermis with cellular infiltration, which, in some cases, extends into the subepidermal tissue. Osborne and Mendel [1921] have shown that, in experiments on a thousand unselected rats, xerophthalmia was only seen in animals supplied with food deficient in fat-soluble vitamin; amongst the latter—the number of animals involved is not stated—the incidence was 50 % and most of the cases developed characteristic symptoms before any marked decrease in body weight had occurred. The administration of a small amount of vitamin A, *e.g.* 0·1 g. of butter fat per animal per day caused a great improvement in, if not the disappearance of, the eye disorder, even though, in some cases, the animal subsequently died.

Another worker [Emmett, 1920] had previously described experiments on 122 rats, 120 of which, or 98 %, developed xerophthalmia.

In view of the discrepancy in the results obtained by these workers, the following details, collected by the author during the course of thirteen experiments upon the vitamin content of various oils and fats, are presented in the hope that they may be of interest. One hundred animals were involved in these experiments: 44 were fed with a fat known to have been deprived of vitamin A by steam-distillation [Stammers, 1921] and the remaining 56 upon fats with varying degrees of deficiency of this vitamin. Of the 44 on the totally deficient diet, 40 were young animals (33–49 days old) at the commencement of the experiments and of these 35 developed keratomalacia between the 31st and 80th days of the experiment. The remaining four were adults (145 days old) when they were used for experiment and they all developed the condition between the 104th and 129th days of the investigation.

Those animals, 56 in number, which were fed upon the partially deficient diets were not so susceptible, 24 only being affected and these between the 35th and 98th days.

From an examination of the appended table, which gives details of the experiments so far as they relate to the incidence of keratomalacia, the following deductions may be made.

	Type of diet	No. of animals in expt.	Average weight and age at commencement	Average weight and age at conclusion	Days of expt. on which keratomalacia appeared	Total affected	Days of expt. on which animals died	Total mortality
1.	Totally deficient	8	77 g. 42 days	82 g. 112 days	35, 45, 49, 52 (2), 56 (3)	8	56, 59, 63 (2), 66 (2), 70 (2)	8
2.	„ „	8	48 g. 33 days	86 g. 124 days	31, 38, 42 (2), 45, 63	6	52, 56 (2), 66, 70, 73, 80	7
3.	„ „	8	58 g. 36 days	70 g. 127 days	38, 42, 45, 49, 59, 66	6	49, 59, 63, 73 (2), 84	6
4.	„ „	8	60 g. 49 days	65 g. 150 days	31, 35, 42, 52, 66, 70, 77 (2)	8	63, 80, 87, 101	4
5.	„ „	8	55 g. 35 days	53 g. 136 days	35, 42, 45, 59, 66, 73, 80	7	66 (2), 84, 87	4
6.	„ „ (adults)	4	214 g. 145 days	174 g. 297 days	104, 108, 122, 129	4	143, 150	2
Total in experiments		44			Total affected	39	Total mortality	31
7.	Partially deficient	8	77 g. 42 days	94 g. 129 days	77	1	—	0
8	„ „	8	48 g 33 days	104 g 124 days	73, 84 (2)	3	—	0
9	„ „	8	44 g 43 days	112 g 144 days	—	0	—	0
10	„ „	8	53 g 49 days	74 g 150 days	35, 49, 56, 63, 66, 80, 98	7	70, 84, 91 (2), 94	5
11	„ „	8	54 g 49 days	61 g. 150 days	66, 77, 80, 94	4	63, 87, 98	3
12.	„ „	8	59 g. 45 days	79 g. 146 days	45, 52, 66, 73, 80	5	87, 98	2
13.	„ „	8	59 g. 45 days	90 g. 146 days	38, 63, 73, 77	4	84, 98	2
Total in experiments		56			Total affected	24	Total mortality	12

The figures in brackets refer to the number of animals.

In the totally deficient group, *i.e.* in the first six experiments, ophthalmic symptoms supervened in 88·6 % of the animals under observation, two-thirds of the cases appearing *before* the 60th day of the experiment, except in the case of the adult animals. In the other group, *i.e.* those on the partially deficient diets, and detailed in the last seven experiments, 42·8 % were affected and two-thirds of the cases appeared *after* the 60th day.

It therefore appears that the susceptibility of the animals and the time of incidence of the disease vary directly with the amount of vitamin A in the fat under investigation, and further that the first factor (susceptibility) is modified by the age of the animal. Thus, adult animals are less susceptible to a deficiency of vitamin A in the diet than are young animals, since the symptoms of the disease were not exhibited in the former until the period between the 104th and the 129th days, but, nevertheless, all four animals were affected and two subsequently died, during the progress of the experiment.

Further, it is interesting to notice the mortality among the two groups. In the first, 31 of the 39 affected animals died and in the second, 12 out of 24. These deaths all occurred during the course of the experiments. No further records were kept of those animals which survived until after the termination of the experiments, so that it is possible that the mortality may have been higher still.

Attention is also invited to the fact that, in the partially deficient group, most deaths occurred among the animals which were provided with the least amount of vitamin A in the diet as evidenced by the growth shown. Thus five deaths occurred in Exp. 10. The average gain in weight in this experiment was only 21 g. over a period of 101 days. Three deaths occurred in Exp. 11. The average gain in weight here was 7 g. over a period of 101 days.

There is one exception to this, viz. Exp. 7, where the average gain over the experimental period of 87 days was only 17 g., but only one animal was affected.

In this latter and in the other experiments on those fats in which a fair quantity of vitamin A was present, no deaths took place and only four cases of ophthalmia, all occurring late in the experimental period, supervened among the 24 animals involved.

VII.

Both cod-liver oil and malt extract have been shown to vary in vitamin content. The following details of experiments carried out upon these products, therefore, may be of interest.

An ordinary commercial sample of combined oil and malt extract was used in conjunction with the basal dietary and suitable allowance was made for the carbohydrate portion of the malt in preparing the food, so that the proper proportions of the dietary constituents should be maintained. The technique of feeding, dosage, weighing, etc., was as previously described in this Journal [Stammers, 1921]. Thus, each animal received about 1·5 g. of fat per day.

Two experiments were undertaken, one (*a*) comparing basal diet plus cod-liver oil and malt extract with basal diet plus a compound consisting of cod-liver oil and malt to which was added a certain quantity of an emulsion of sterilised baker's yeast and some neutralised lemon juice. This is spoken of as "compound V." The other experiment (*b*) is a comparison of basal diet plus compound V with basal diet plus steam-distilled palm-kernel oil (*i.e.* a diet deprived of vitamins).

Sixteen animals were used in each test, eight for each preparation and the technique was as previously described.

The first experiment consisted of two sets of eight rats fed upon (*a*) basal diet plus compound V and (*b*) basal diet plus cod-liver oil and malt. No controls were used, as it was merely desired to make a comparison between the two preparations. The animals, in this case, averaged 48 g. at the beginning of the experiment and were 36 days old. The test was continued for

91 days and, during this time, the compound V animals gained 141 g. and the cod-liver oil and malt animals 105 g. The normal gain for this period and age is 146 g.

In the second experiment, the animals used were 45 days old at the commencement, but they were exceptionally well grown and averaged 79 g. in weight. This test was carried on for 70 days and, during this time, the gain in weight was 146 g. The normal gain over this period for animals of this age is 123 g. The animals deprived of vitamins gained slightly during the first ten days, but afterwards they lost weight rapidly and at the 31st day had all succumbed.

The accompanying graphs (Fig. 2) show the growth recorded and Donaldson's normal curves corresponding to the ages of the animals used.

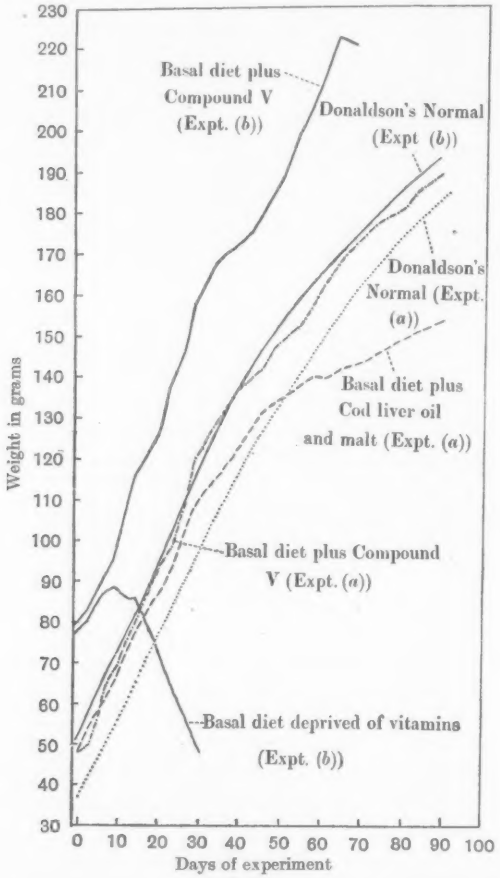

Fig. 2.

Summary and Conclusions.

Cod-liver oil and malt extract gave very satisfactory growth which was improved by the addition of yeast and lemon juice. It appears certain that "cod-liver oil and malt extract" contain sufficient vitamins A and B to provide the necessary stimulus to growth when the animals receive 1·5 g. of oil per day. (This represents about 8 g. of malt in the combined extract, which was substituted, in making up the dietary, for the starch and sugar usually supplied.)

If further supplies of vitamins B and C are added, in the forms of yeast and lemon juice respectively, better growth still is recorded. It is concluded that this may be due to the increase in vitamin B supplied by the yeast.

I am indebted to Messrs Lever Brothers, Limited, for permission to publish these researches.

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III. ON THE POSITION OF THE OPTIMUM TEMPERATURE OF THE ACTION OF A FERMENT OR LYSIN.

By JOHN BROWNLEE.

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(*Received October 22nd, 1923.*)

As the question of the optimum temperature of the action of a ferment has not, so far as I have seen, been mathematically treated, the following solution is offered.

Generally it is found that the coefficient of increase in the rate of a chemical action with temperature is given to a first approximation by Arrhenius' expression

$$e^{\mu\left(\frac{1}{T_0}-\frac{1}{T}\right)},$$

where T is the absolute temperature and T_0 the absolute temperature chosen as zero in the experiments. For a short range of temperature, however, this can be sufficiently approximately expressed by a coefficient of the form

$$e^{\mu(T-T_0)},$$

which lends itself more easily to mathematical manipulation.

Let then the rate at which the ferment acts at T_0 be λ and the temperature factor $e^{\nu T}$, so that the rate of action of the ferment at any temperature is

$$\lambda e^{\nu T}.$$

Further, assume that the ferment at a temperature T_0 is destroyed at a rate K, that the temperature factor is

$$e^{\mu T}$$

and the rate of destruction at any time

$$Ke^{\mu T}.$$

Let f denote the amount of the ferment present at any time. Then

$$\frac{df}{dt} = -Ke^{\mu T}f,$$

whence

$$f = ae^{-Kte^{\mu T}}, \qquad \text{.....(1)}$$

where a is the amount of ferment when $t = 0$.

The fermentable substrate may be considered as altered according to the law of a unimolecular reaction. If the initial amount be A and the amount

fermented at any time t be x and if the rate of action of the ferment is proportional to its amount, it follows from equation (1) that

$$\frac{dx}{dt} = a\lambda e^{\nu T} e^{-tKe^{\mu T}}, \qquad \text{.....(2)}$$

which on integration becomes

$$-\log (A - x) = C - \frac{a\lambda}{K} e^{T(\nu-\mu)} e^{-tKe^{\mu T}}.$$

If $x = 0$ when $t = 0$ we have

$$C = -\log A + \frac{a\lambda}{K} e^{T(\nu-\mu)},$$

so that

$$\log (A - x) = \log A - \frac{a\lambda}{K} e^{T(\nu-\mu)} + \frac{a\lambda}{K} e^{T(\nu-\mu)} e^{-Kte^{\mu T}}. \qquad \text{.....(3)}$$

If an optimum action exist for a given temperature, $A - x$ must be a minimum, *i.e.* $\frac{d \log (A-x)}{dT}$ is zero.

It is obvious that equation (3) involves the time as an essential constituent so that the solution obtained is not the optimum temperature for action of the ferment but the optimum temperature for a specified duration of time.

The differentiation gives

$$-\frac{a\lambda}{K}(\nu - \mu) e^{T(\nu-\mu)} - a\lambda\mu t e^{T(\nu-\mu)} e^{-Kte^{\mu T}} + \frac{a\lambda}{K}(\nu - \mu) e^{T(\nu-\mu)} = 0$$

or

$$(\nu - \mu)\{1 - e^{-Kte^{\mu T}}\} + K\mu t e^{\mu T} = 0. \qquad \text{.....(4)}$$

If $Kte^{\mu T}$ be noted by ξ this becomes

$$(1 - e^{-\xi}) = \frac{\mu}{\mu - \nu} \xi e^{-\xi}.$$

As ξ is essentially positive there is no optimum unless $\mu > \nu$.

The equation has only one real positive root, say r. Thus

$$Kte^{\mu T} = r$$

or

$$T = \frac{1}{\mu} \log \frac{r}{K} - \frac{1}{\mu} \log t. \qquad \text{.....(5)}$$

Having started with four constants, it is to be observed that only two are found in the final equation, which thus becomes an empirical relationship and provides the means of determining only one of the specific values of the constants of reaction on which the reasoning is based. From equation (4) it is obvious that the value of r depends on μ, ν and K, and that from equation (5) only $\frac{r}{K}$ can be determined. Thus μ is the only one of the original constants which survives.

As an example of this formula compared with the facts, the only observations I have found which are sufficiently extensive to compare are those given by Walbum [1915] with reference to the optimum temperature of an *Epeira* lysin. The observations are reproduced in the accompanying table. In order that the optimum temperature with each time of action should be as far as possible independent of personal equation a parabola of the third degree

was fitted to each set of observations. The fitting was done to the observations between 10° and 40° for the experiments which lasted from 10 to 20 minutes and between 0·2° and 32·6° for the experiments which lasted for one hour or more. These observations having been fitted, the maximum was determined in the usual way and a fitting made to equation (5). The results obtained from the experiment are shown in the accompanying table with the theoretical fitting obtained by least squares in parallel columns. Considering the probable error of the optimum obtained from the observations, this must be considered a very good fit.

Table showing the percentages of haemolysis in a series of experiments of different duration and at different temperatures.

Time	0·2°	10·3°	19·0°	27·4°	32·6°	40·4°	45·3°
10 minutes	0	2	12	15	17	10	0
20 „	0	8	19	23	20	11	2
1 hour	7	25	35	30	25	17	9
2 hours	17	35	40	35	30	19	11
5 „	27	48	50	42	35	25	15

Optimum temperature.

Duration of experiment	Actual	Theoretical
10 minutes	29·8	29·3
20 „	26·6	26·4
1 hour	20·5	21·7
2 hours	18·6	18·8
5 „	15·7	15·0

REFERENCE.

Walbum (1915). *Z. Immunitätsforsch.* Orig **23**, 623.

IV. NOTE ON THE EFFECT OF SODIUM TRICHLOROACETATE ON THE REDUCTION OF COPPER SOLUTIONS BY GLUCOSE.

By DAVID STIVEN.

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(*Received October 22nd, 1923.*)

As a preliminary to some investigations on glucose, it was thought necessary to test the effect, if any, of protein precipitants on the accuracy of glucose estimations. The quantitative methods examined were the Bertrand method and the micro-method of Schaffer and Hartmann [1921]. It was observed that when trichloroacetic acid was used to precipitate the proteins and the glucose in the filtrate determined by either of the above methods, the percentage of glucose found was always very much lower than that of a control, done on a glucose solution not treated with trichloroacetic acid. This discrepancy was traced to the presence of sodium trichloroacetate which can inhibit the reduction provided the salt be present in sufficiently great concentration.

The experiments quoted below were done on the Schaffer-Hartmann micro-method to try and find the cause of this result.

The results of Exp. 1 show the extent of this inhibiting effect.

Exp. 1. 3 cc. of a 0·05 % glucose solution were used. Water was added to bring the total volume up to 5 cc.

N/10 trichloroacetate added cc.	Cuprous oxide found mg.
0	2·39
1·0	0·99
1·5	0·26
2·0	0·23

5 cc. of the Schaffer copper solution, heated with 5 cc. of water, required 19·4 cc. *N*/200 thiosulphate. The same volume of thiosulphate was required by another control—not heated—but with 5 cc. of *N*/10 trichloroacetate in place of the water.

The presence of the trichloroacetate in a concentration of 0·02 *N* so inhibits the reduction that there is no visible formation of cuprous oxide, although the titration indicates a very small reduction of the copper. The cupric copper of the control is unaffected by the presence of trichloroacetate.

Polarimetric evidence of a reaction between the glucose and trichloroacetic acid is entirely negative. Moreover as it was noticed that the appearance of the cuprous oxide, if formed, is retarded when the trichloroacetate is present, experiments were performed to see if any evidence could be obtained, indicating a reaction between the copper and the trichloroacetate.

Exp. 2. Four samples of the following mixture were made up: 5 cc. of Schaffer copper solution + 1·5 cc. of sodium trichloroacetate $N/10$ + 0·5 cc. of water.

This mixture was heated for various periods on the water bath prior to adding the glucose. After the addition of the 3 cc. of glucose solution, heating was continued for another 15 minutes.

	Cuprous oxide found mg.
1. Heated for 5 minutes before addition of glucose	1·49
2. „ 10 „ „ „	2·89
3. „ 15 „ „ „	3·21
4. Not heated	0·22

That it is the undecomposed trichloroacetate which is active is shown in Exp. 3. The reduction can be stopped at any stage after it has started, by adding the trichloroacetate. The cuprous oxide already formed is not destroyed.

Exp. 3. 3 cc. glucose solution + 2 cc. water heated with 5 cc. of the Schaffer copper solution. At various stages of the reduction 0·5 cc. of a 0·5 N solution of trichloroacetate was added.

Time of adding trichloroacetate	Cuprous oxide found mg.
After 5 minutes reduction	2·54
„ 10 „ „	3·08
„ 15 „ „	3·37
No trichloroacetate added	3·41

These results seem to indicate that there occurs a reaction between the copper of the Schaffer solution and the sodium trichloroacetate by which the copper passes into a non-reducible complex. This organo-copper compound decomposes gradually on heating—like sodium trichloroacetate itself; but during the heating the glucose will be destroyed by the sodium carbonate of the copper solution and no reduction—or very little—will result, even after the copper has reverted to its reducible condition.

Doughty [1919] and Doughty and Freeman [1922] have obtained evidence of an organo-copper complex of trichloroacetic acid but its behaviour on heating with glucose in alkaline solution has not been investigated.

Since the above experiments were made, the author has found a reference to this effect of trichloroacetic acid, in a paper by Denigès [1922] in which he states that this acid "apporte des perturbations profondes sur le pouvoir réducteur des sucres." Beyond this observation no investigations seem to have been made with a view to elucidating the mechanism of the effect.

Conclusions.

The use of trichloroacetic acid as a protein precipitant prior to glucose estimations by any of the copper reduction methods is to be avoided. There is experimental evidence of the formation of an unstable organo-copper compound of trichloroacetic acid in which the copper is non-reducible.

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V. ESTIMATION OF SUGAR IN THE BLOOD.

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(*Received October 9th, 1923.*)

The principal object of this note is to point out a fallacy occurring in this estimation as commonly practised.

As in the course of our experiments we found that certain details must be very carefully attended to if correct results are to be expected, it will be convenient to mention these first.

Four years ago Folin and Wu [1919] published a method for the estimation of blood sugar in which the cuprous oxide formed by reduction of alkaline copper tartrate by the sugar was converted into a deep blue solution by means of a reagent containing phosphomolybdates and phosphotungstates in strongly alkaline solution. This method was criticised on the grounds (1) that the cuprous oxide had opportunity to be partially oxidised by the air before the blue colour was formed; (2) that the blue colour due to any unreduced copper tartrate introduced an error which could not be allowed for; (3) that the phosphotungstic-phosphomolybdic reagent gives a blue colour with the so-called phenols of blood.

To meet these objections Folin and Wu [1920] modified their method (1) by conducting the reduction in constricted test-tubes, thus lessening the possibility of atmospheric oxidation, and (2) by using a new reagent which does not react with phenols, but does give a blue coloration with cuprous oxide. As this reagent is used in acid solution, any error due to unreduced copper tartrate is eliminated. It is this newer method which has been used by numerous recent workers, including Calvert [1923].

In working with it we soon noticed that duplicate standards made at the same time often differed from one another very considerably. It was at first thought that this might be due to the standards not having been prepared absolutely at the same instant, but although we find that the blue colour fades appreciably with time, the difference is not perceptible when the interval of time is small. A standard made one hour after another one is identical with it, but one made three hours afterwards is distinctly darker. This change of tint with time has been noticed by others [Fleury and Boutot, 1923]. In using this method it is therefore necessary to perform the colorimetric measurement within an hour of the time at which the blue colour is produced.

A factor of much greater importance, which turned out to be the explanation of the observed differences, is the temperature of the liquid at the moment when the blue colour is formed. The colour is darker the lower the temperature. Folin and Wu seem to be aware of this, for they recommend that the tubes be immersed in cold water for two or three minutes to bring them to the same temperature before adding the reagent, but they do not emphasise the point as being of the great importance we have found it to be, and many of those who have used the method in one modification or another are in the habit of adding the reagent to the hot solution. It is impossible to obtain concordant results if this be done, for the temperature of the last of the batch of tubes is bound to be very much lower than that of the earlier ones. The length of time for which it is necessary to keep the tube in the cold bath will depend, of course, on the size of the tube, and must be determined for any given quantities by seeing, with a thermometer, how long it takes. We use very small tubes, which at the material time contain only 2 cc. of solution, and with these we find an immersion of $3\frac{1}{2}$ minutes in water at about 20° to be necessary. With larger tubes the time will be considerably longer.

The following experiment indicates the magnitude of the difference caused by different temperatures. Two dextrose solutions containing the same amount (20 cmgm.) of dextrose were treated exactly as described by Calvert [1923], except that in one case the reagent was added immediately after the tube was removed from the boiling water, whilst in the other the addition was made after the tube had been cooled in water at about 20° for five minutes. On comparing in the dilution colorimeter it was found that, taking the "cold" tube as standard (20), the "hot" tube had a value of only 15. The shades of colour were so different as to make more than an approximate comparison impossible. The colour produced at the higher temperature is an indigo blue, quite different from the pure deep blue obtained in the cold.

We can confirm the necessity of using tubes with a constriction, and the results quoted below show that no re-oxidation of cuprous oxide then occurs.

In our hands, therefore, the process takes the following form. The solutions are those described by Folin and Wu in their second paper. 1 cc. of the solution containing dextrose and 1 cc. of alkaline copper tartrate are placed in a small test-tube such that 2 cc. come to the middle of the constriction. A standard is similarly prepared from 1 cc. of pure dextrose solution containing 0·1 mg., or, as we prefer to term it, 10 centimilligrams (abbreviated cmgm.) of pure dextrose. The tubes are heated in a large, vigorously boiling water-bath for six minutes. They are supported in a cage of wire gauze made so that they do not actually touch the bottom of the bath. At the end of six minutes the tubes are immersed in a beaker of water at room temperature for $3\frac{1}{2}$ minutes. 1 cc. of Folin's reagent is next added to each tube and, after a pause of two minutes, 3 cc. of water. After mixing, 5 cc. is pipetted off from each tube and compared in the dilution colorimeter. Should the un-

known contain much dextrose, a smaller quantity, such as 2 cc. or 1 cc., is taken for the colorimeter. The following results show the degree of accuracy obtained.

Table I.

Taken	Found	Taken	Found
5·0	5·32	17·5	17·6
7·5	7·52	20·0	21·0
10·0 (standard)	—	25·0	25·0
12·5	12·56	30·0	30·2
15·0	14·8	40·0	40·85

There is for any coloured substance at a given thickness of layer a particular concentration at which the colour comparison is most sensitive. Using a 10 mm. layer we find the most sensitive point is in the neighbourhood of the blue solution resulting from 10 cmgm. of dextrose, and for that reason we use it as standard. Below that concentration the solution pales very rapidly, and this is why the estimation of the 5 cmgm. sample in Table I shows a bigger error than most of the others. This is, however, a case that does not often occur in practice, as by suitably arranging the quantity of unknown material taken it can nearly always be ensured that the unknown solution shall be stronger than the standard. The actual colorimetric comparison then takes place at the concentration of the standard. At this point a difference of 1 % can be detected with certainty, that is, the addition of one drop (0·05 cc.) of water to 5 cc. of solution makes a perceptible difference. This being so, some explanation is required of why some of these results show an error of as much as 5 %. In all cases where the error has exceeded 1 or 2 %, it is such that the amount found is *more* than it should be, and we think the only explanation of these discrepancies is in the occasional contamination of tubes, pipettes or the like with dust of organic origin. In the worst case in Table I the absolute value of the error is only 1 cmgm., and it is easy to imagine enough contamination to account for that. The tubes, pipettes etc. are kept clean with distilled water and dried in a hot oven. Periodically they are treated with chromic acid. Drying with alcohol and ether should be avoided at all costs.

One other observation deserves mention. As Calvert remarks [1923], a mixture of equal volumes of the alkaline copper tartrate and phosphomolybdate solutions should give a colourless liquid. Now, we find that, although the mixture is at first colourless, a blue coloration gradually develops on standing. It is first noticeable after a few minutes, and after an hour or two it is quite marked. We are unable to suggest any explanation for this phenomenon, but the exactness of the results given in Table I shows that, if anything of the kind happens to the unreduced copper tartrate in the course of an ordinary estimation, the error occasioned is too small to matter.

A fallacy in Calvert's results. In his paper already cited, Calvert is chiefly concerned with replacing the standards actually prepared from glucose by suitably selected Lovibond Tintometer glasses. In this way the labour involved

in continually making up fresh standards is avoided. In test experiments with this arrangement, using a series of dextrose solutions of known dextrose content, Calvert observed that the amounts of dextrose found did not agree with the amounts taken. Plotting his results on a curve he found that at concentrations below that of the standard the values found were less than they should be, whilst at concentrations above that of the standard the values found were greater than they should be. The error so involved is very considerable, especially when the unknown solution is much stronger or weaker than the standard. For instance, using Calvert's units of measurement, when 0·05 is found, the real value should be about 0·075, that is, the result is 33 % too low. Similarly when 0·1 is found, the real value is 0·125 (error 20 %). Calvert's standard is 0·2 in his units, and there, for obvious reasons, the value found agrees with the true value. Above that point the divergence steadily increases with concentration. A "found" value of 0·4 corresponds with a true value of 0·34 (error 18 %). A found value of 0·5 corresponds with a true value of 0·4 (error 25 %). A found value of 1·0 corresponds with a true value of 0·7 (error 40 %).

Calvert supposes these differences to be due to the fact that the copper is not proportionately reduced, and his procedure therefore is to convert his "found" values into true values by reference to the curve. The results quoted in Table I above, however, show that the copper *is* proportionately reduced. Indeed, in view of the small quantities dealt with, the agreement is remarkable.

It remains to give an explanation of Calvert's results. As it is more than ten years since one of us [Stanford, 1913] pointed out that *all* colorimeters of the Duboscq type were liable to give very inaccurate results excepting in the special case where the concentrations of known and unknown were nearly equal to begin with, we thought at first that Calvert's curve was merely an elegant demonstration of the errors of his colorimeter, but the figures given in Table II below show that this is not the case. It appears that, in this particular instance, the colorimetric errors [Stanford, 1913] happen to be equal in amount as well as opposite in direction, at any rate in the neighbourhood of the standard. As we happen to have a Duboscq instrument, we made

Table II.

	Found		
	Duboscq colorimeter		Dilution
Taken	at 10 mm.	at 15 mm.	colorimeter
5·0	5·15	5·15	4·95
7·5	7·41	7·50	7·40
10·0 (standard)	—	—	—
12·5	12·8	12·7	12·4
15·0	16·4	14·8	14·8
20·0	21·4	19·8	19·6
25·0	26·3	25·9	25·0
30·0	31·2	30·6	30·9
40·0	41·7	41·7	39·95
50·0	50·0	49·7	49·5

experiments with that. A series of dextrose solutions of known concentration was prepared, ranging from 5 to 100 cmgm. per cc. The colorimetric comparisons of the blue solutions were made

(*a*) with the Duboscq colorimeter, using a 10 mm. layer for the standard;

(*b*) with the same, using a 15 mm. layer as standard;

(*c*) with the dilution colorimeter.

The results are shown in Table II.

Reference has already been made to the important influence of the temperature at which the blue colour is produced on its intensity and shade. To examine the possibilities of this source of error, the experiments shown in Table III were made. The figures in the columns marked 1, 2 and 3 were obtained by following Calvert's procedure exactly, that is, by adding the reagent to the hot solution. The figures in the columns marked 4, 5 and 6 were obtained by the same procedure, excepting that the reduction mixture was cooled to about 20° before the reagent was added. In each case the colorimetric comparison was performed in three ways:

(*a*) with Duboscq colorimeter and daylight;

(*b*) with Duboscq colorimeter illuminated by a 100 w. half-watt lamp having a piece of "Dalite" glass in front of it;

(*c*) with the dilution colorimeter.

Table III.

	Hot			Cold		
Taken	Duboscq daylight	Duboscq Dalite glass	Dilution colorimeter	Duboscq daylight	Duboscq Dalite glass	Dilution colorimeter
100	—	143	148	115	107	113
80	120	111	108	100	94	97
60	86	81	74	79	73	74
40	49	47	47	46	46	43
20	Standard					
Curves	1	2	3	4	5	6

These figures are plotted in Fig. 1. In this diagram the curve marked *TT* is the true curve showing that the values found are the same as those taken. It is therefore a straight line inclined at 45°. The data plotted are those of Table I, and the curve shows that the values found agree with the quantities taken within the limits of experimental error.

The curve marked *CC* is Calvert's curve, transposed from his paper. In between this curve and the straight line will be seen the six curves resulting from Table III. As to these it may be remarked that No. 1, obtained with the Duboscq and daylight, hot, approximates very closely to Calvert's curve.

All the Duboscq curves show that the colorimetric comparison is noticeably affected by the nature of the source of light used, as was to be expected. Actually, the comparisons were more exact with the electric lamp and "Dalite" glass than with genuine daylight.

At this point it still remained to explain the reason for Calvert's curve.

It seemed possible that the time of heating had to do with it. Accordingly,

a series of experiments was undertaken in which the time of heating was increased to 12 minutes, instead of the usual six. The curve numbered 12 (Fig. 1) resulting from these experiments is intermediate between Calvert's

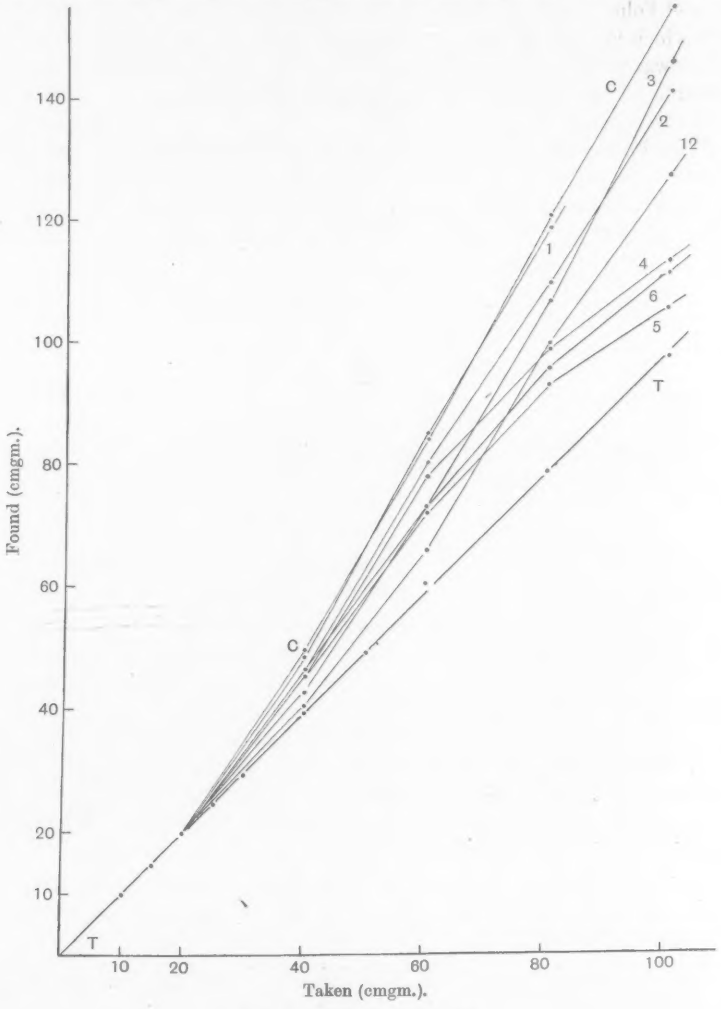

Fig. 1. Dextrose estimations.

curve and the true one, but it shows that the real source of error is not to be found there.

The most striking feature of Calvert's careful work is that, at concentrations above the standard, he always found *more* than he took, whilst at

concentrations *less* than the standard, he found *less* than he took, and yet our experiments shown in Table I prove that the reaction is quantitative.

The only other difference between Calvert's conditions of reaction and those of Folin and of us is in the matter of concentration. In the experiments from which he drew his curve, Calvert (apart from doing it in the hot, more or less) was working with solutions which in respect of both the alkaline copper tartrate and the Folin reagent were very much weaker than those used by Folin and by us.

Experiments increasing the amount of reagent showed no difference from the results of the method as used by Calvert. When, however, the amount of alkaline copper tartrate was increased (in proportion) to that used by Folin and by us, quantitative results were obtained, as shown in Table IV.

Table IV.

Dilution colorimeter Taken	Calvert's standard 20 Found
20	Standard
40	39
60	59
80	80
100	100

This proves that the curve given by Calvert is based on a fallacy and is due to his not having had a sufficient concentration of the alkaline copper tartrate. It would appear that at concentrations in the neighbourhood of his standard the reaction is not quantitative unless a large excess of the alkaline copper tartrate is present. When the relative quantities given by Folin, and used by us, are employed, accurate results are obtained.

Tests of the method in the conditions which we have indicated above show that added dextrose is then quantitatively recovered from a blood filtrate, and also that the content in reducing sugar of a blood filtrate remains sensibly unchanged after two days.

Summary.

1. The blue coloration obtained by the action of phosphomolybdic acid on cuprous oxide in acid solution fades with time.

2. The temperature at which the blue coloration is produced must be the same for the standard and for the unknown to be compared with it.

3. The comparisons must be carried out in a dilution colorimeter.

4. The reacting substances (especially the tartrate) nust not fall below the concentrations given by Folin.

The cost of this work was in part defrayed from a grant by the Medical Research Council, to whom our thanks are due.

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Folin and Wu (1919). *J. Biol. Chem.* **38**, 106.
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VI. MECHANISM OF OXIDATION IN THE PLANT. PART II.

AN INVESTIGATION OF SUBSTANCES WHICH ARE CAPABLE OF BEHAVING AS PEROXYDASES.

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(*Received September 10th, 1923.*)

The question of peroxide formation in plant tissues has been considered in a previous paper [Gallagher, 1923]. There remains to be considered the mechanism by which the oxygen of peroxides may be rendered active for purposes of oxidation. This leads to the study of the enzyme peroxydase.

Peroxydase is characterised by its ability to oxidise guaiaconic acid and other oxydase reagents in presence of peroxide. Pure peroxydase preparations have no oxidising action on these reagents in the absence of peroxide; likewise, the peroxide is relatively inactive as an oxidising agent in the absence of peroxydase. It has been pointed out by Wolff [1908] that the behaviour of peroxydase in this respect is similar to that of certain salts of copper and of iron. Woker [1914] states that formaldehyde and acetaldehyde are capable of behaving as peroxydases and catalases. The very frequent presence of iron in peroxydase preparations has led to the belief that this metal in some form is the active constituent of the enzyme, a view which recalls the suggested connection of manganese with oxydases of the laccase type. Woker, as a result of her observations, suggests that peroxydases are substances of aldehyde nature.

The preparation of oxidising enzymes which have been shown to be free from manganese or iron [Rosenfeld, 1906; de Stoecklin, 1907; Bach and Tcherniak, 1908; Bach, 1910, Willstätter and Stoll, 1918 and Van der Haar, 1921] would appear to indicate that the mineral constituents of oxydase preparations are not the main active components of the enzyme. Rather does it appear likely that the chief active enzyme constituent is a substance of organic nature.

As a preliminary to the study of the chemical nature of naturally occurring peroxydase an examination was made of a variety of compounds which are capable of replacing peroxydase in the oxidation of guaiaconic acid by peroxide. The object of the study was to determine the nature of the reaction

by means of which the oxygen of the peroxide is set free in an active condition. It was also sought to determine whether there existed among compounds which behave as peroxydases a common peculiarity of structure to which their reactivity in this respect might reasonably be ascribed.

The Peroxydase Reaction of Aldehydes.

The peroxydase and catalase reactions of formaldehyde and acetaldehyde led Woker [1914] to the conclusion that naturally occurring peroxydases, catalases and reductases are due to the same agent which is a substance of aldehyde character. Benzaldehyde, it was stated, showed no activity as a peroxydase until after repeated rectification, and no catalase action was observed in the case of this aldehyde.

An investigation which was carried out in the present work on the behaviour of a number of aldehydes towards a mixture of guaiacum tincture and peroxidised turpentine led to the conclusion that the peroxydase reaction is generally associated with aliphatic aldehydes. No specimen of benzaldehyde examined appeared to have any action on a mixture of guaiacum and peroxide, but samples of propyl, *n*-butyl, *n*-valeric and aldehydes, as well as oenanthaldehyde, formaldehyde and acetaldehyde, were found to give distinct peroxydase reactions. These were the only aliphatic aldehydes examined. It was found, however, that different specimens of the same aldehyde frequently reacted to a markedly different degree with a mixture of guaiacum and turpentine. While some laboratory specimens of formaldehyde, for instance, gave a strong blue colour with a mixture of guaiacum solution and turpentine, others, of apparently similar concentration and purity, when tested under comparable conditions, gave but a faint coloration. These differences of behaviour admit of two explanations: either the specimens of low reactivity contain traces of a substance which inhibits the reaction with guaiacum, or else the peroxydase reaction is due to an impurity in the aldehyde and not to the aldehyde itself. The above-mentioned aldehydes were consequently rectified. In no instance did the fresh distillate show any reactivity towards a mixture of guaiacum and peroxide, but in each case the peroxydase reaction was found to reappear on storing the aldehyde in an unstoppered flask for several days. Specimens of the freshly distilled substances which were stored out of contact with air in sealed tubes failed to acquire a peroxydase reaction. It is thus evident that the peroxydase reaction commonly associated with aliphatic aldehydes is due to the presence of an impurity which is produced by the action of air on the aldehyde.

Nature of the substance to which the peroxydase reaction commonly given by aliphatic aldehydes is due.

The fact that by the action of air on many aliphatic aldehydes a substance is produced which is capable of oxidising guaiaconic acid in presence of peroxide, points to the likelihood that this reactive substance is an oxidation

product of the aldehyde. The aldehydes in question are known to be fairly easily oxidised in the air to the corresponding acids. It is necessary to remember that not alone are the acids incapable of inducing the oxidation of guaiacum but they tend to inhibit it. Clearly, therefore, the substance with which we are concerned must be an oxidation product intermediate between the aldehyde and the acid, or, which seems less likely, an oxidation product bearing no relation whatsoever to the acid. The facts of the case recall the problem of the autoxidation of benzaldehyde. In connection with this substance it has been fairly definitely established that a substance of peroxide nature is formed as an intermediate product in the oxidation to the acid. There is this essential difference, however; benzaldehyde appears to form a peroxide which is capable of oxidising guaiaconic acid in presence of peroxydase, while the substances derived from the aldehydes under consideration are only capable of replacing the peroxydase in the oxidation of guaiaconic acid by an added peroxide such as turpentine peroxide or ethyl hydroperoxide. The peroxide of benzaldehyde accordingly appears to be constituted similarly to hydrogen peroxide, while the substance formed from aliphatic aldehydes appears to be directly related in some way to peroxydase. Baeyer and Villiger [1900] have prepared a peroxide of benzaldehyde by treating the substance with hydrogen peroxide. Aliphatic aldehydes do not readily form peroxides with hydrogen peroxide [Langheld, Weyl, 1914].

The only known substances of peroxide nature derived from aliphatic aldehydes are the so-called monoperoxides or ozonides described by Harries and Langheld [1906]. The aldehyde monoperoxides are formed when the aldehyde is treated with ozone. One atom of oxygen is added on per molecule of aldehyde, and the formula at present accepted for these substances is $R.CH=O=O$. That the peroxydase reaction of the aldehydes under consideration is very probably due to a substance of this type is shown by the following experiment.

Exp. A. One gram of pure freshly rectified oenanthaldehyde was placed in a tube immersed in a good freezing-mixture and a current of ozonised oxygen from a single Siemen's induction tube was passed through the liquid at the rate of about one bubble per second for half an hour. The generation of ozone was then stopped and the current of oxygen was continued at a somewhat faster rate for about fifteen minutes to remove traces of uncombined ozone. The contents of the tube were then poured into 20 cc. of well-cooled rectified spirit. A few drops of bromocresol purple were added as indicator and addition of *N* alkali was made, drop by drop, till the solution changed to a green colour. A little of the neutralised solution was now poured into two test-tubes, one of which contained a fresh alcoholic solution of guaiaconic acid and the other a mixture of guaiaconic acid solution and 1·5 % ethyl hydroperoxide. In the second tube a blue colour soon appeared which became quite intense in a few minutes. The tube which contained guaiaconic acid alone only began to develop a faint blue colour after 20–30 minutes. Since guaiaconic acid is

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readily oxidised by free ozone the latter test is essential to ensure that no free ozone be present in the aldehyde solution.

In making the above experiment it is necessary to keep the ozonised aldehyde well cooled when neutralising; otherwise the ozonide is rapidly decomposed by the excess of free acid or alkali as the case may be.

The experiment was repeated with *n*-valeric aldehyde with similar results. Control experiments in which ordinary oxygen was passed through a quantity of the aldehyde under similar conditions gave practically no coloration with guaiaconic acid and ethyl hydroperoxide.

Discussion.

This experiment seems to offer strong proof that the peroxydase reaction given by the aldehydes examined is due, not to the aldehyde itself, nor to a compound of the aldehyde with hydrogen peroxide as Woker suggests, but to a relatively uncommon class of peroxides—a monoperoxide or ozonide. Harries and Langheld [1906] represent the structure of the substance formed by the action of ozone on oenanthyl aldehyde by the formula

$$CH_3 . (CH_2)_5 . CH : O : O.$$

The formation of substances of this type among organic compounds seems to be characteristic of the carbonyl group.

The association of aldehyde with peroxydase enzymes.

In view of the association of the peroxydase reaction with specimens of the common aldehydes it is interesting at this stage to note certain observations made on the possible aldehyde nature of natural peroxydase.

(i) Preparations of peroxydase made by alcoholic precipitation from some common vegetables (potato, horse radish and mangold) give a strong coloration with Schiff's reagent. If a section of mangold root or potato tuber be treated with Schiff's reagent, the location of the coloration which takes place is the same as when a section is treated with tincture of guaiacum and peroxide. The reaction in both cases takes place at the tracheides and at the epidermis.

(ii) Peroxydase preparations obtained by alcoholic precipitation likewise give an intense reaction for aldehyde when treated with diazobenzenesulphonic acid and sodium amalgam in presence of alkali [Fischer and Penzoldt, 1883].

(iii) The peroxydase solutions examined likewise give Lewin's reaction for aldehydes [Cohn, 1916]; when a peroxydase solution is added to a mixture of piperidine and sodium nitroprusside solution a blue colour develops. This changes to bluish-green on adding glacial acetic or dilute hydrochloric or sulphuric acid. The original blue colour changes to rust-colour on adding strong mineral acids, and the blue colour is restored on dilution. A rust-colour is likewise obtained on adding caustic soda to the original solution.

The possible aldehyde nature of plant peroxydases will be dealt with further in a subsequent paper on the enzyme itself.

Peroxydase Activity of Metallic Oxides.

Bertrand [1897] in his investigation of laccase suggested that the activity of this substance is due to its manganese content. He found that all the salts of manganese which he examined were capable of causing the oxidation of guaiacum in presence of oxygen. He observed that the salts of weak acids had considerably greater oxidising power than those of strong acids, and concluded that the active substance was manganous oxide formed by hydrolysis. It is known that the presence of free acid markedly inhibits the action of oxydases and peroxydases. For this reason, and in view of the fact that the peroxydase activity of aldehydes has been shown to be due to a substance of oxide nature, the present study of peroxydase activity among inorganic compounds has been confined chiefly to metallic oxides. The results are recorded in the table given on p. 34.

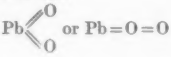
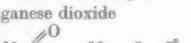
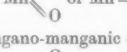
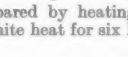
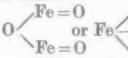
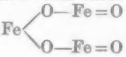
The tests were made by adding a small quantity of the oxide (sufficient to cover the tip of a pen-knife blade) to a few cc. of a 1 % solution of guaiaconic acid in 50 % alcohol. Each oxide was tested with the reagent alone, and in presence of a drop of 2·5 % hydrogen peroxide. The test in each case was repeated with indophenol mixture (1 % *p*-phenylenediamine and 1 % *α*-naphthol in 50 % alcohol). Only such colorations are recorded (p. 34) as were really distinct, appearing in at least one minute.

The peroxides of calcium, barium, strontium, sodium and potassium had no action on guaiaconic acid with, or without, hydrogen peroxide, but in presence of a substance which behaves as a peroxydase it was found that these peroxides, when added in small quantities, were capable of replacing hydrogen peroxide in the oxidation of guaiaconic acid. The general structural formula given for these peroxides is .

In each case where an oxide was found to behave as an oxydase or peroxydase a distinct evolution of oxygen from hydrogen peroxide was also found to take place.

Discussion.

Relatively little of a really decisive nature appears to be known of the structural formulae of inorganic substances. Most of the formulae given in the tables, however, are those generally accepted for the oxides in question. Metallic peroxides fall into two main classes corresponding to the ozonides and antozonides of Schönbein or the polyoxides and superoxides of Mendeléef. Schönbein's ozonides include the peroxides of the heavy metals and his antozonides those of the alkali metals and of the alkaline earths. The former, it was assumed, were derived from ozone and the latter from hydrogen peroxide. It is worthy of note in the present connection that the metallic oxides which are capable of oxidising guaiaconic acid, with or without addition of hydrogen peroxide, are those of the metals which form peroxides of the so-called ozonide or polyoxide type. On the other hand, Mendeléef's

Oxide	Guaiaconic acid	Guaiaconic acid and H_2O_2	Indophenol mixture	Indophenol mixture and H_2O_2
Copper.				
Cupric oxide Cu=O	No action	Coloration	No action	Coloration
Lead.				
Lead monoxide Pb=O	No action	Coloration	No action	Coloration
Lead dioxide	Coloration	—	Coloration	—
Manganese.				
Manganous oxide Mn=O. Prepared by heating carbonate in a current of nitrogen	Coloration on brief shaking with air	No coloration	No coloration	Coloration
Manganese dioxide	Immediate coloration	—	Immediate coloration	—
Mangano-manganic oxide  Prepared by heating MnO_2 at a white heat for six hours	No action	No action	No action	No action
Iron.				
Ferrous oxide Fe=O. Prepared by heating iron in a current of carbon dioxide	No action	Coloration	No action	Coloration
Ferric oxide 	No action	No action	No action	No action
Magnetic iron oxide 	No action	Coloration	No action	Coloration
Nickel.				
Nickelous oxide Ni=O. Prepared by heating nickel carbonate in a current of nitrogen	No action	No action	No action	Coloration
Nickelic oxide  Prepared by gently heating nickel carbonate in air	Immediate coloration	—	Immediate coloration	—
Cobalt.				
Cobaltous oxide Co=O. Prepared by heating cobalt carbonate in current of nitrogen	No action	Coloration	No action	Coloration
Cobaltic oxide  Prepared by gently heating cobalt carbonate in air	Immediate coloration	—	Immediate coloration	—
Cobalto-cobaltic oxide  Prepared by heating cobalt carbonate at white heat for six hours	No action	Coloration	No action	Coloration

superoxides or Schönbein's antozonides are found to be capable of replacing hydrogen peroxide in the guaiacum reaction. When we consider the formulae of the oxides (such as cupric oxide, lead monoxide, ferrous oxide, ferroso-ferric oxide, cobaltous oxide and cobalto-cobaltic oxide) which behave as peroxydases, it will be seen that the common feature in each is an oxygen atom linked thus, = O. It has already been pointed out that this is the type of oxide group which characterises the active derivatives of aldehydes.

The behaviour of manganous oxide in the foregoing experiments deserves special notice. When added to a solution of guaiaconic acid a coloration rapidly appears on shaking with air in the absence of hydrogen peroxide. When hydrogen peroxide is present the guaiaconic acid is not oxidised, while a rapid decomposition of the peroxide takes place with evolution of oxygen. It is known that hydrogen peroxide in neutral or alkaline solutions readily transforms manganous hydroxide into the dioxide which catalytically decomposes the hydrogen peroxide. Although manganese dioxide alone is capable of immediately oxidising guaiaconic acid it is found that in presence of excess of hydrogen peroxide only the catalytic interaction of the dioxide and hydrogen peroxide takes place. If guaiaconic acid be present it is not attacked by the dioxide under these conditions. Thus, if a small quantity of manganese dioxide be placed in a test-tube and a couple of cc. of 2·5 % hydrogen peroxide be added, guaiaconic acid quickly added afterwards is not oxidised. In support of the above explanation of the behaviour of manganous oxide it is found that if an aqueous solution of ethyl hydroperoxide be used in place of hydrogen peroxide, manganous oxide immediately causes the oxidation of guaiacum. With organic peroxides, therefore, manganous oxide seems capable of behaving as a peroxydase.

The relative ease with which manganous oxide causes the oxidation of guaiaconic acid in presence of air is no doubt due to the facility with which the manganous oxide oxidises in air to manganese dioxide. The dioxide, as will be seen above, is itself capable of oxidising guaiaconic acid.

The marked difference between the manner in which manganous oxide reacts towards guaiaconic acid and that in which it behaves towards indophenol mixture remains to be considered. No coloration is obtained with indophenol mixture in the absence of hydrogen peroxide. The inhibiting influence of phenolic substances on autoxidation has been referred to in a previous paper [Gallagher, 1923]. There appears to be little doubt that the presence of α-naphthol in the reagent inhibits the autoxidation of the manganous oxide. This explanation is supported by the observation that if manganous oxide be added to guaiaconic acid solution containing a little α-naphthol no oxidation of guaiaconic acid takes place on shaking with air. Addition of a little quinol or catechol was likewise found to prevent the oxidation of guaiaconic acid by manganous oxide. It was noted that the phenol itself gradually underwent oxidation when present in the mixture. On the other hand indophenol mixture, unlike guaiaconic acid, is oxidised by manganous

oxide in presence of hydrogen peroxide. This appears to be due to the fact that indophenol mixture is much more easily oxidised by hydrogen peroxide than is guaiacum, and a relatively weaker stimulus is required to accelerate the oxidation. The case of nickelous oxide is a further illustration of the difference between guaiaconic acid and indophenol mixture in this respect.

The apparent inhibitory influence of phenolic substances on the autoxidation of manganous oxide recalls observations made in the previous paper of this series in regard to the action of these substances on peroxide formation in plant tissues. The question arises as to whether the action of oxygen on manganous oxide results in the introduction of a peroxide group similar to that formed in the case of lecithins, terpenes and other common autoxidisable substances. The peroxides formed in such cases appear to be structurally related to hydrogen peroxide. They are capable of replacing hydrogen peroxide in testing for peroxydases. Does manganese dioxide, for instance, contain the group $\begin{matrix}-O\\|\\-O\end{matrix}$ which appears to be characteristic of peroxides of the hydrogen peroxide type? As already pointed out, it is formed in the action of hydrogen peroxide on manganous oxide. Lead dioxide, which resembles manganese dioxide in oxidising properties, is likewise formed from the monoxide by the action of a neutral or alkaline solution of hydrogen peroxide. Cupric oxide reacts with hydrogen peroxide to form an unstable dioxide of the formula CuO_2, H_2O or CuO, H_2O_2. This compound is probably structurally represented by

$$Cu{=}O\begin{matrix}OH\\||\\OH\end{matrix} \quad \text{or} \quad O{=}Cu\begin{matrix}OH\\\\OH\end{matrix}$$

The evidence suggests that the dioxides of manganese and lead in their hydrated forms are of similar structure.

The present survey of the subject, therefore, suggests that for the oxidation of substances such as those in use as oxydase reagents the presence of two different types of oxides is essential: (i) the ozonide or mono-peroxide type, = O, characteristic of peroxydases and (ii) the hydrogen peroxide type, $\begin{matrix}-O\\|\\-O\end{matrix}$, characteristic of the so-called "oxygenases." These may, or may not, be found in the same plant constituent. The behaviour of manganous oxide is an illustration of how they may both be found in the same molecule. In a study of the catalytic decomposition of hydrogen peroxide Kastle and Loevenhart [1903] concluded that the first stage in the decomposition is the formation of an unstable additive higher oxide. It appears likely that some such additive compound is also the active oxidising agent in oxydase reactions, *e.g.*

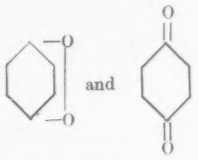
$$R_1{=}O + \begin{matrix}O\\|\\O\end{matrix}\!\!>R_2 \rightarrow R_1{=}O\begin{matrix}O\\||\\O\end{matrix}\!\!>R_2.$$

The structural relationship of a substance of this type to ozone is significant

in view of the fact that ozone readily oxidises the reagents employed in testing for oxydases.

Organic substances capable of oxidising guaiaconic acid.

Two organic substances which possess the property of oxidising guaiacum directly have been met with in the course of the present work, viz. quinone and benzoyl peroxide. It is interesting to note that a certain amount of controversy has existed as regards the constitution of these two substances. Quinone has been variously represented as



Benzoyl peroxide has been given the formulae:

$$\begin{matrix} C_6H_5 . CO . O \\ | \\ C_6H_5 . CO . O \end{matrix}$$

[Willstätter and Hauenstein, 1909]

and

$$\begin{matrix} C_6H_5 . CO \\ \\ C_6H_5 . CO \end{matrix} \!\!>\!\! O = O$$

[Rius y Miró, 1920]

In view of the foregoing conclusions, the direct oxidation of guaiacum by these substances would suggest that each is a tautomeric mixture of both forms.

Conclusions.

The observations made on the peroxydase activity of inorganic oxides suggest that the presence of one or other of the heavy metals, such as manganese or iron, in a plant may undoubtedly have a direct bearing on oxydase activity. In this respect the relation between the behaviour of manganous oxide and the known activity of direct oxydases is significant. Iron, in the state of oxide, only appears to influence the oxidation of guaiaconic acid when in the ferrous condition. The inactivity of ferric oxide seems to favour the closed formula, $Fe\langle\begin{matrix}O\\O\\O\end{matrix}\rangle Fe$. X-ray spectra of ferric oxide likewise support this formula. On the other hand it has been observed that ferric salts frequently behave as peroxydases. It is known that ferric salts, even of strong acids, readily undergo hydrolysis with production of the corresponding oxysalts:

$$Fe\langle\begin{matrix}\overline{A}\\\overline{A}\\\overline{A}\end{matrix} + H_2O = Fe\langle\begin{matrix}O\\\\\overline{A}\end{matrix} + 2H\overline{A}.$$

Here again, we have production of the oxide group typical of ferrous oxide.

Nonetheless there exists much evidence that the chief enzyme constituent in the plant is a substance which is separable from mineral matter. The aldehyde reactions given by partially purified peroxydase preparations, as already quoted, seem to be of considerable significance in this respect. In a study of the natural enzyme, experiments have been carried out which

appear to throw considerable light on the nature of peroxydase and its relation to the various types of substances studied in the present paper.

SUMMARY.

A study is made of a series of substances which, like the enzyme peroxydase, are capable of activating the oxygen of hydrogen peroxide and of similarly constituted peroxides.

It is shown that peroxydase activity is commonly found to be associated with aliphatic aldehydes. It is not a property of the pure aldehyde, but is due to a substance of oxide nature which is formed by the action of atmospheric oxygen. This oxide appears to be analagous with the monoperoxide formed by the action of ozone.

A series of reactions is given which show that the peroxydase enzyme preparations obtained by alcoholic precipitation contain a substance or substances of aldehy[illegible]ure.

The influence of [illegible] metallic oxides on the oxidation of guaiaconic acid and of indophen[illegible]re by peroxide was studied. The property of inducing the oxidation [illegible]eagents is found to be fairl[illegible] generally associated with derivatives [illegible]lled heavy metal.

The results in general [illegible]ica[illegible] peroxydases are characterised by the [illegible]nce o[illegible] group of the type = O. Such a group, in conjunction wi[illegible]ne ordinary [illegible]oxide group in which oxygen is linked to oxygen, $\begin{matrix}-O\\|\\-O\end{matrix}$, gives rise to a higher oxidation potential than that possessed by either group alone. It is suggested that as a preliminary stage in the oxidation combination takes place between these two types of oxide yielding a substance of oxidation potential comparable to that of ozone:

$$R_1{=}O\begin{matrix}O\\\|\\O\end{matrix}R_2.$$

The author wishes to thank the Royal Commissioners for the Exhibition of 1851 for the grant of a Scholarship.

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VII. MECHANISM OF OXIDATION IN THE PLANT. PART III.

PEROXYDASE. OBSERVATIONS ON THE THERMOSTABILITY OF THE PEROXYDASE OF THE MANGOLD.

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From the Department of Biochemistry, Cambridge.

(*Received September 10th, 1923.*)

In a previous communication [Gallagher, 1924] an examination was made of various substances which, like the enzyme peroxydase, have the property of activating the oxygen of peroxides. The results of this study indicate that the activity of such substances is associated with the presence of an oxide group of the type R = O. A group of this type appears to be present in the monoperoxides of aliphatic aldehydes and, as a rule, in the oxides of the heavy metals. In the present paper the investigation is extended to the natural enzyme with a view to determining its possible relationship to the compounds already examined.

The peroxydase chosen for special study was that of the mangold root. The manner of preparing the enzyme by the method of alcoholic precipitation has already been described [Gallagher, 1923].

Peroxydase possesses properties which appear to justify its classification with typical enzymes. Its activity is destroyed on heating, though as a rule a higher temperature is usually required for its complete destruction than is the case with most other enzymes. Its power is also markedly influenced by variation of hydrogen potential, and it is readily precipitated by the common enzyme precipitants. Although, as Kastle and Loevenhart [1901] have pointed out, oxidising enzymes are practically uninfluenced by such enzyme poisons as mercuric chloride and silver nitrate, traces of such substances as hydroxylamine, hydrocyanic acid or aniline, are known to have a marked inhibiting influence. Hydroxylamine in the dilution of 1 : 70,000 was found to have a quite definite influence on a solution of mangold peroxydase.

Action of heat on the peroxydase of the mangold.

If a few cc. of fresh mangold juice be brought to boiling for two minutes and if, on cooling, it be immediately tested with guaiacum and peroxide, the peroxydase is found to have been destroyed. A somewhat remarkable feature of the juice of this root is that if it be allowed to remain in the cold after undergoing the above treatment it is found to regain its activity. Specimens of peroxydase obtained by alcoholic precipitation behave in a like manner. The present work deals chiefly with a study of this reactivation.

Comparative colorimetric method for estimating peroxydase.

In the first place it was sought to determine quantitatively in what manner and to what extent reactivation took place after a certain degree of heating. For this purpose a relatively rapid method of estimation was essential. A colorimetric method was adopted which was based on the use of guaiaconic acid. This substance appears to be the most generally sensitive of oxydase reagents. It possesses the disadvantage, however, of yielding a relatively unstable colouring matter. In consequence of this it is found impossible to prepare standard solutions of the oxidation product for comparison. Euler and Bolin [1909] recommend indigo blue as a standard. This was found unsatisfactory. The standard used was a 20 % solution of copper nitrate to which 2 cc. of saturated cobalt nitrate was added per 100 cc. of solution. The colour of this solution matches that of the oxidation product of guaiacum extremely well. Yet again there was the disadvantage that while guaiaconic acid is insoluble in water, peroxydase is insoluble in alcohol. This difficulty was overcome by allowing the reaction to take place in 50 % alcohol.

The following reagents are required in dealing with peroxydase solutions approximating in strength to that of natural juices:

(i) A 2·5 % solution of *guaiaconic acid* in 50 % alcohol.

(ii) A stock solution of pure neutral 2·5 % *hydrogen peroxide.*

(iii) A standard solution of *peroxydase* containing a concentration of enzyme not more than about twice that of natural plant juices such as those of the potato or the mangold.

(iv) *A standard tint* consisting of a 20 % solution of copper nitrate to which a saturated cobalt nitrate solution has been added in the ratio of 2 cc. of cobalt nitrate for every 100 cc. of copper solution.

It is first necessary to prepare standardisation curves with these reagents by comparing the colour of certain definite thicknesses of the standard tint with colorations given under specified conditions by different dilutions of the peroxydase solution. Convenient dilutions of the peroxydase solution for this purpose are 1, $\frac{1}{2}$, $\frac{1}{3}$, $\frac{1}{5}$, $\frac{1}{10}$, $\frac{1}{20}$. The procedure adopted in carrying out the oxidation of guaiaconic acid consists in first adding 10 cc. of 50 % alcohol to a 50 cc. measuring flask. 1 cc. of guaiaconic acid solution is then added, followed by 1 cc. of hydrogen peroxide solution, and finally by 1 cc. of

peroxydase solution. The time at which the peroxydase is added is noted and the contents of the flask are gently shaken for one minute, after which addition of rectified spirit to the 50 cc. mark is quickly made. After thoroughly mixing the contents of the flask the solution is allowed to stand for a further five minutes in a dark place. The colour of the solution is then quickly compared with that of the copper-cobalt solution in a colorimeter. This procedure is repeated with the different dilutions of the peroxydase solution and the colorations obtained as compared with, say, 1 cc. of the standard tint, are plotted on a curve against the various strengths of peroxydase used. It is advisable to take readings with two or three different thicknesses of the standard tint with each solution so that the values obtained from the different curves may be checked against one another.

With reagents of the strengths given above measurable differences of coloration are obtained by variations of one or two per cent. in the peroxydase strength of the juice of common vegetables. If it be necessary to deal with more concentrated preparations these must first be suitably diluted before accurate readings can be obtained with reagents of the above concentration.

It will be seen that the special value of this method in dealing with the reactivation of peroxydase after heating is that the strength of the reactivated solution relative to that of the original solution may readily be determined. To arrive at reasonable accuracy it is necessary that the estimations be always carried out under the same conditions of treatment and temperature, etc.

Extent to which the reactivation of mangold peroxydase takes place under varying conditions.

Using the quantitative method just outlined, the extent to which a solution of mangold peroxydase underwent reactivation after different degrees of heating was determined. The specimen used required a little less than two minutes at 100° for its total temporary inactivation. About 10 cc. of the solution was added to a boiling-tube by means of a pipette. The tube was then immersed in boiling water. The solution was well stirred by means of a thermometer, and when a temperature of 100° was attained the time was noted and heating at this temperature was continued for one minute. While continuing to stir the solution the tube was then immersed in cold water for one minute. The activity of the peroxydase present was then estimated. Similar readings were taken at suitable intervals during six hours. The experiment was repeated with specimens of the same peroxydase solution which had been heated in a similar manner for three and five minutes, respectively. The progress of the reactivation is shown in all three cases in Fig. 1. The strengths of the reactivated solutions are expressed in percentages of that of the original solution.

It will be seen that after heating to 100° for one minute the activity of the peroxydase is reduced to 9 % that of the original solution. After standing

at room temperature for six hours this same solution had increased in activity to 40 % that of the original. After heating for three minutes no measurable coloration was obtained with guaiacum at the beginning. After standing for six hours the strength of this solution was 14 % that of the original, while after heating for five minutes the final strength attained was 10 %.

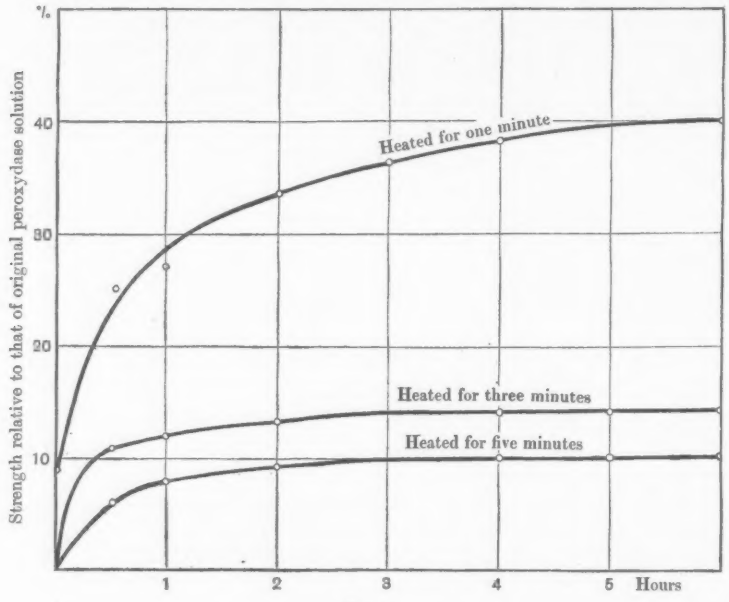

Fig. 1.

When a specimen of peroxydase solution, subsequent to heating, was stored in a sealed tube, reactivation appeared to take place to the same extent as when air had free access.

Discussion.

The reaction curves show that the rate of reactivation is greatest at the beginning and that it falls off considerably after the first hour. Equilibrium is attained more readily the longer the period of heating has been. The evidence suggests that the reaction is bimolecular and that one of the substances taking part undergoes decomposition on prolonged heating. An explanation which suggests itself at this stage is that we are dealing with the interaction of a zymogen and a second substance which is capable of transforming it into peroxydase.

Before proceeding to an account of some experiments which appear to throw some light on the nature of the substances to which the regeneration of per-

oxydase is due it is necessary to recall results arrived at in an investigation of substances which are capable of behaving as peroxydases [Gallagher, 1924].

It was shown that the aldehyde monoperoxides and various oxides of the heavy metals were the main types of synthetic compounds with which the property of oxidising guaiacum in presence of peroxide is found to be associated. It was observed that the preparations of peroxydase examined gave (*a*) Schiff's reaction, (*b*) Fischer and Penzoldt's and (*c*) Lewin's reactions for aldehyde. The test for aldehyde is found to remain positive after the peroxydase is temporarily destroyed by heating. The elements of the heavy metal class sought for in the peroxydase solutions under examination were manganese and iron. Manganese was found to be practically absent from preparations from the mangold root, and it was found that the iron content of solutions from this root, prepared as already described, was usually in the region of 1 mg. of iron per 100 cc. of the solution. An observation made to the effect that traces of iron added to aldehydes, such as formic or valeric, results in a distinct increase in peroxydase activity, would seem to be of especial interest in connection with the present problem.

Influence of iron on the peroxydase activity of aliphatic aldehydes.

In the quantitative treatment of this subject a colorimetric method of analysis was employed which was similar to that used in dealing with the plant enzyme. Certain modifications, however, were found to be essential. The active derivatives of the common aldehydes are incapable of carrying out the oxidation of guaiacum in presence of much hydrogen peroxide. An organic peroxide must therefore be used. Ethyl hydroperoxide was chosen, chiefly on account of its solubility.

Note on the use of ethyl hydroperoxide as a reagent for peroxydases.

Although hydrogen peroxide has been made use of in previous sections of this work, subsequent experience proves that an aqueous solution of ethyl hydroperoxide is very much to be preferred, not only in quantitative, but also in qualitative work with oxydases. Its action very probably approximates more nearly to reactions in the plant cell than does that of hydrogen peroxide. Unlike most organic peroxides this reagent is fairly soluble in water and it does not appear to be affected by catalase. Unlike hydrogen peroxide it does not seem to have much injurious effect on peroxydase, even when present in excess. It is found to be sensitive, not alone in dealing with weak solutions of peroxydase but also in the investigation of peroxydase substances of relatively weak character such as the solutions of iron and aldehyde at present under investigation. The use of hydrogen peroxide in the estimation of relatively strong peroxydases is only justified when the method used is strictly comparative.

The curves shown in Fig. 2 were obtained by adding to 10 cc. of 50 % alcohol (*a*) 1 cc. of 1·5 % aqueous ethyl hydroperoxide, (*b*) 1 cc. of 2·5 % guaiaconic acid solution and (*c*) 2 cc. of the mixture of aldehyde and an iron salt of a weak acid (ferric oxalate). The mixture, after shaking, was allowed to stand for five minutes and a further 10 cc. of 50 % alcohol was added before taking the colorimetric readings. The readings were taken after suitable intervals against the same copper-cobalt solution as previously used during

six hours in all. The standardisation curves were prepared immediately at the end of the experiment by quickly taking readings from various dilutions of the solution corresponding to curve 1. The strength of the solution after six hours was given the value 100, and the other readings are expressed in percentages accordingly.

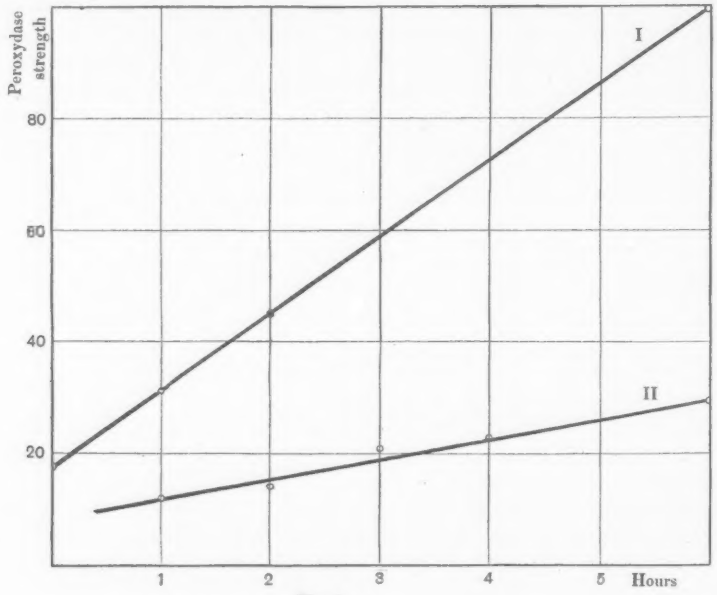

Fig. 2.

I 20 % formaldehyde containing 3·5 mg. Fe per 100 cc.
II 20 % „ „ 1·125 „ „

It will be seen that the rate of increase in peroxydase activity is roughly proportional to the amount of iron present. The curve of the reaction in each case is practically a straight line. A 1 % solution of formaldehyde containing 3·5 mg. of iron per 100 cc. was found to increase in activity somewhat more rapidly than the solution containing the same amount of iron with 20 % formaldehyde. Since the aldehyde used contained a small amount of free acid this unexpected difference is no doubt partly due to the lesser quantity of acid in the weaker aldehyde solution. Fig. 3 shows the behaviour of weak solutions of formic and valeric aldehydes in presence of iron. In making the colorimetric determinations in this case 1 cc. only of the peroxydase solution was employed as compared with 2 cc. in the case of the solutions previously dealt with. The method of estimation was otherwise similar to that used in preparing Fig. 2.

The p_H of the formaldehyde solution, as determined colorimetrically at the commencement of the experiment was found to be 4·6 and that of the valeric aldehyde solution 4·4. These values appeared to vary but little throughout

the experiment. A special feature of the experiment made with valeric aldehyde is the manner in which the reaction falls off after three hours. This aldehyde ' but slightly soluble in water, so that the half-saturated aqueous solution contains a relatively small quantity of the substance in solution. This would appear to explain the attainment of an early equilibrium.

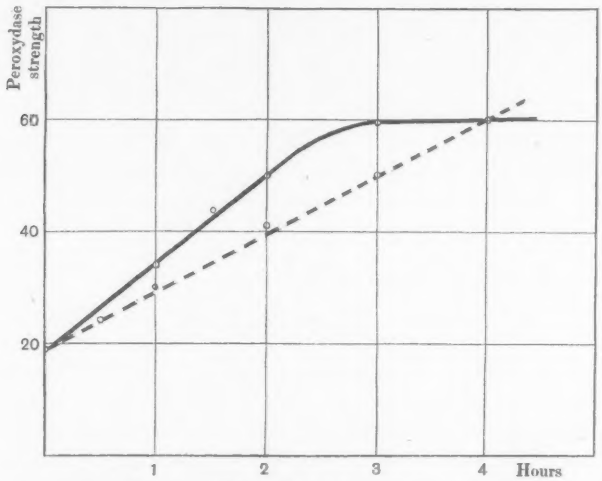

Fig. 3. – – – – represents 1 % formaldehyde containing 1·75 mg. of iron per 100 cc. solution.
——— represents a half-saturated aqueous solution of *n*-valeric aldehyde containing 1·75 mg. of iron per 100 cc. solution.

Discussion.

In view of the observations already recorded that mangold peroxydase solutions, even after destruction of the peroxydase, contain a substance of aldehyde nature as well as an iron content of the same order as that used in dealing with formic and valeric aldehydes, the above experiments appear to constitute a remarkable parallel to the reactivation of the natural enzyme after its destruction by heat. The fact that the interaction between formaldehyde and iron is linear in the instances studied may be explicable on the grounds that such a small quantity of active product is formed that the relative proportions of the interacting substances do not vary appreciably. A more likely explanation for the linear nature of the curves lies in the fact that excess of this aldehyde was used in all cases, while the iron probably acts catalytically. In the case of valeric aldehyde a lower ratio of the aldehyde was employed and the rate of activation decreased after three hours. Here, it would seem, we have attained a condition of affairs more nearly approximating that of the enzyme solution. It has already been shown [Gallagher, 1924] that the derivative of aldehydes which is responsible for the peroxydase

reaction is very probably a substance of ozonide or mono-peroxide nature, R . CH = O = O. In the formation of this substance iron probably acts as an oxygen carrier. Whether the action is due to ferric compounds only has not yet been definitely determined. Specimens of ferrous oxalate were found capable of activating formaldehyde, but here, it will be realised, the problem of entirely excluding ferric compounds is extremely difficult.

It is proposed to investigate further the influence of mineral substances on the peroxydase activity of aldehydes with a view to determining whether the interaction described constitutes, in all respects, a true parallel to the plant enzyme.

SUMMARY.

The destruction of the peroxydase of the mangold on heating is shown to be of temporary character. Reactivation of the enzyme is found to take place when the solution is subsequently allowed to remain some time in the cold. The progress of the reactivation has been studied quantitatively by means of a comparative colorimetric method.

The peroxydase solutions examined were found to contain iron. They also gave several reactions for aldehyde. The aldehyde character of the solution was maintained after the peroxydase had been temporarily inactivated by heat.

It is shown that traces of iron added to aqueous solutions of aliphatic aldehydes give rise to an increase of peroxydase activity on standing in the cold.

The most probable explanation for the reactivation of solutions of the enzyme after heating is that the zymogen or precursor of the enzyme is an aldehyde and that the enzyme is produced by the catalytic action of iron.

The author wishes to thank the Royal Commissioners for the Exhibition of 1851 for the grant of a Scholarship.

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VIII. STUDIES IN EXPERIMENTAL TETANY.

III. ON ALKALOSIS AND ACIDOSIS.
IV. ON THE HYDROGEN ION CONCENTRATION OF THE BLOOD.
V. ON THE ALVEOLAR CARBON DIOXIDE TENSION.

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(Rockefeller Foundation.)

(*Received October 10th*, 1923.)

The problem, the results of the investigation of which are here presented, was to determine the changes in the acid-base balance of the blood in experimental tetany and to attempt to decide what was the essential nature of these changes; to arrive at a conclusion as to the extent of the change, if any, in the reaction of the blood; and to note the tension of CO_2 with which the arterial blood was in equilibrium in the lungs. The further question of the variability in the acidity of the haemoglobin molecule was also borne in mind as an integral part of the problem, but is not here discussed.

III. The question of the associated conditions of alkalosis and acidosis in tetany.

The question as to whether or not tetany is associated with a condition of alkalosis has been a subject of investigation for some considerable time, the general trend of opinion being that alkalosis may be produced by parathyroid extirpation, intravenous injections of sodium bicarbonate solutions, gastric lavage and hyperpnoea. The question, however, as to whether this condition of alkalosis is due to an uncompensated alkali excess or an uncompensated CO_2 deficit has not been answered. It can only be answered by an investigation of the time relations of the changes in the acid-base balance of the blood occasioned by any of the above mentioned procedures.

That parathyroid extirpation results in a condition of alkalosis, an alkalosis due to increase in base, was suggested by Wilson and others [1915] and McCann [1918]. On the other hand the work of Hastings and Murray [1921] and that of Underhill and Nellans [1921] would indicate that there was no change in the alkalinity or in the CO_2-combining power of the blood in parathyroid

tetany. In a recent paper Greenwald [1922] states that tetany produced by the administration of sodium bicarbonate is not due to alkalosis but is due to the toxic effect of large doses of sodium salt, this effect being essentially of the nature of a sudden and marked disturbance of the relationship between the sodium ion and other cations. This idea has also been emphasised by Collip [1921].

After a long series of experiments upon the reaction of the blood of dogs and rabbits following parathyroid and thyroparathyroid extirpation, we came to the conclusion that a high p_H was not a necessary concomitant of parathyroid tetany and that the condition of alkalosis, due to increased production of alkali in the blood, if it existed, did so to a comparatively small extent, and that only during the short period preceding the acute attack of tetany; and further, the results obtained suggested that the condition of alkalosis was due, not to an uncompensated increased production of alkali, but to an uncompensated increased loss of acid. Since it was also noted that, with the appearance of those marked physical signs of tetany to which reference has already been made [Cruickshank, 1923], the p_H, which had increased slightly, fell to, and passed below, the normal figure, we had further to conclude that the acute tetany was associated with a condition, in which the reaction of the blood changed from the alkaline to the acid side of normality. It was also thought that the muscular tremors, which in many cases were very marked indeed, might by the production of acid bodies be responsible for a definite alteration from alkalosis to acidosis in the associated blood condition, and that they constituted a sign of the commencement of a new phase of the condition produced by lack of parathyroid substance. As considerable emphasis has in the past been laid upon the fact that tetany is a condition associated with alkalosis, with a high CO_2-combining power of the blood with a p_H as high as 7·8, it was deemed expedient, in order to decide to what extent this change to the alkaline side of the normal occurred, to secure data on the acid-base balance of the blood and to determine the hydrogen ion concentration of the blood during the period after operation in which no signs of tetany were present, or at least no excessive respiratory movements, no marked muscular tremors and no sickness. It was to prove or disprove these ideas that the present work was undertaken.

Methods.

As in previous work, total thyroparathyroidectomies were performed. Blood samples were drawn, under cocaine, from the femoral arteries, and as the femoral arteries can be readily exposed at any point in their course, blood could be obtained with ease on at least four consecutive occasions. The blood for the immediate estimation of the total CO_2 by the Van Slyke method [Van Slyke and Stadie, 1921] was drawn under liquid paraffin, in a small centrifuge tube coated with potassium oxalate to make a 0·3 g. % solution; the blood to be equilibrated with various tensions of CO_2 was collected in a

50 cc. centrifuge tube, also coated with oxalate; both tubes were placed in a rack surrounded with ice. A little difficulty was experienced in obtaining potassium oxalate which gave a reaction between p_H 7·0 and 7·4; the oxalate supplied by Merck, Darmstadt, was found to be satisfactory. For obtaining approximate carbon dioxide tensions in the tonometers, the gas manifold described by Austin and others [1922] was used. To obtain the necessary equilibration data, four large and four small tonometers, connected by a short rubber tube, were used; the capacity of each of the former being 300 cc., that of the latter 20 cc. Ten cubic centimetres of blood were drawn into each of the large tonometers, equilibration was carried out at 38°, the period of equilibration extending for not more than 40 minutes. After equilibration the blood samples were sealed with mercury in the small tonometers and the estimation of their CO_2 content proceeded with immediately, while the air, with which the blood had been equilibrated, was sealed with mercury in the large tonometers and set aside in a water-bath at room temperature (28°–32°) for subsequent examination with the latest form of the Haldane-Henderson [Henderson 1918] gas analysis apparatus.

Calculations. The abbreviations used are those suggested by Van Slyke and others [1922]: namely mM for millimolecular, $[CO_2]$, $[H_2CO_3]$, $[BHCO_3]$ for their respective millimolecular concentrations, p_{CO_2} for the CO_2 tension in millimetres of mercury. From the equilibration data a curve, relating $[CO_2]$ or $[BHCO_3]$ to p_H, was constructed. By fixing four points to obtain a curve which has an approximately linear character, it is possible to determine with a considerable degree of accuracy the position of the curve, and by interpolating upon this curve the $[CO_2]$ value of the blood as drawn, the p_H of the circulating blood is determined. From this p_H and the known $[CO_2]$, the $[BHCO_3]$, $[H_2CO_3]$, p_{CO_2} and alveolar CO_2 were calculated by use of Hasselbalch's [1917] equation,

$$p_H = p_{K_1} + \log \frac{BHCO_3}{H_2CO_3},$$

where $p_{K_1} = 6{\cdot}18$. Other equations used in the calculations are:

$$[BHCO_3] = \frac{[CO_2] \times R}{(R+1)},$$

$$p_{CO_2} = \frac{[CO_2]}{0{\cdot}03\,(R+1)} \text{ or } p_{CO_2} = \%\,CO_2\,(B - 49)$$

where $$R = \frac{BHCO_3}{H_2CO_3} \text{ and } \log R = p_H - p_{K_1},$$

$$[H_2CO_3] = \frac{\alpha\,CO_2 \times p_{CO_2}}{760 \times 0{\cdot}0224}$$

$$= 0{\cdot}03 \times p_{CO_2}$$

where $$\alpha = 0{\cdot}511 \text{ [Bohr, 1905]}.$$

Changes in the acid-base ratio have been noted, while all the changes in $[H_2CO_3]$ and $[BHCO_3]$, p_H and alkali reserve have been related as percentage changes from their respective normal amounts. Changes in the alkali reserve are measured at the normal p_H, because at a constant p_H the change in the

blood bicarbonate expresses directly the change in excess of base over acids, other than H_2CO_3. Since equilibration data were obtained in all these experiments, one could, by extrapolation of the $[CO_2]$ values of the blood drawn upon each succeeding day to the value which would obtain at the normal p_H, calculate the corresponding $[BHCO_3]$ value and thus determine directly the change in the alkali reserve.

Discussion of Results.

It has been the general result in this laboratory to find all animals develop signs of tetany in the course of the second or third day after operation. This then afforded the opportunity to determine what was the blood condition existing prior to the development of symptoms of acute tetany, and for this purpose the acid-base balance, the p_H, and the alkali reserve of the blood were determined 24, 48 and 72 hours after operation.

Table I.

Dog No. I. Tetany on third day after operation.
Fine tremor of muscles of head, neck, shoulder girdle and abdomen.
Respiration rate—200 per minute, with apnoeic pauses.

Time in hrs.	Atmosphere % CO_2	Atmosphere p_{CO_2}	Alveolar air CO_2	Blood % CO_2	Blood $[CO_2]$	$[H_2CO_3]$	$[BHCO_3]$	R	p_H	Remarks
0	5·503	39·33	5·17	40·95	18·28	1·180	17·10	14·45	7·34	Normal
24	3·630	25·30	3·29	31·75	14·16	0·760	13·40	17·38	7·42	Tetany *A*
					16·20		15·15			At normal p_H
48	3·23	22·66	2·98	27·40	12·23	0·680	11·55	19·05	7·46	Tetany *B*
					14·30		13·38			At normal p_H

	Tetany *A* Total changes	Tetany *A* % changes	Tetany *B* Total changes	Tetany *B* % changes
p_H	= 0·08 +	1·09	= 0·12 +	1·63
p_{CO_2}	= 14·03 –	—	= 16·67 –	—
$[BHCO_3]$	= 3·70 –	21·62	= 5·55 –	32·45
$[H_2CO_3]$	= 0·420 –	35·60	= 0·50 –	42·40
Alkali reserve	= 1·95 –	11·40	= 3·72 –	21·75

Table II.

Dog No. II. Tetany on third day after operation—very mild.
Muscular tremors very faint.
Respiration rate—120 per minute.

Time in hrs.	Atmosphere % CO_2	Atmosphere p_{CO_2}	Alveolar air CO_2	Blood % CO_2	Blood $[CO_2]$	$[H_2CO_3]$	$[BHCO_3]$	R	p_H	Remarks
0	4·710	47·33	6·22	51·80	23·12	1·420	21·70	15·14	7.360	Normal
24	4·470	31·66	4·18	38·00	16·97	0·940	16·03	17·02	7.412	Tetany *A*
					18·25		17·12			At normal p_H
48	4·430	31·33	4·12	39·40	17·59	0·940	16·65	17·78	7.430	Tetany *B*
					19·10		17·92			At normal p_H

	Tetany *A* Total changes	Tetany *A* % changes	Tetany *B* Total changes	Tetany *B* % changes
p_H	= 0·062 +	0·842	= 0·07 +	0·951
p_{CO_2}	= 15·67 –	—	= 16·00 –	—
$[BHCO_3]$	= 5·67 –	26·11	= 5·05 –	23·25
$[H_2CO_3]$	= 0·48 –	33·77	= 0·48 –	33·77
Alkali reserve	= 4·58 –	21·11	= 3·78 –	17·42

Table III.

Dog No. III. Tetany developed on fourth day after operation.
Legs spastic, with muscular twitching of hind legs. Tremor produced by moving, as in lifting, the dog.

Time in hrs.	Atmosphere % CO_2	Atmosphere p_{CO_2}	Alveolar air CO_2	Blood % CO_2	Blood $[CO_2]$	Blood $[H_2CO_3]$	Blood $[BHCO_3]$	R	p_H	Remarks
0	4·320	30·33	3·99	36·70	16·380	0·910	15·47	17·18	7·415	Normal
24	3·730	26·33	3·47	33·92	15·150	0·790	14·36	21·88	7·520	Tetany *A*
					16·602		15·69			At normal p_H
48	3·216	32·66	2·98	31·62	14·12	0·68	13·44	19·95	7·48	Tetany *B*
					15·75		14·88			At normal p_H

	Tetany *A* Total changes	Tetany *A* % changes		Tetany *B* Total changes	Tetany *B* % changes
p_H	= 0·105 +	1·55	p_H	= 0·065 +	0·87
p_{CO_2}	= 4·00 −	—	p_{CO_2}	= 3·67 −	—
$[BHCO_3]$	= 1·110 −	7·12	$[BHCO_3]$	= 2·03 −	13·11
$[H_2CO_3]$	= 0·12 −	13·18	$[H_2CO_3]$	= 0·23 −	25·29
Alkali reserve	= 0·220 +	1·42	Alkali reserve	= 0·59 −	3·83

In Tables I, II and III are given details of the results obtained on three dogs in which the tetany was of a comparatively mild character. The first of these dogs showed fine tremors of the abdominal and shoulder muscles and marked contractions of the neck and head muscles on the third day after operation, while in the others the tremor was much less marked. The respiration rate increased in the first to a maximum of 200 per minute; in the other two the maximum did not exceed in either case 120 per minute. There seems to have been a delay in the development and a diminution in the severity of the symptoms in these animals which may be accounted for by the daily withdrawal of 60 cc. of blood. It will be seen that in the case of animals 1 and 2 there is a definite fall in the alkali reserve in the first 24 hours, a

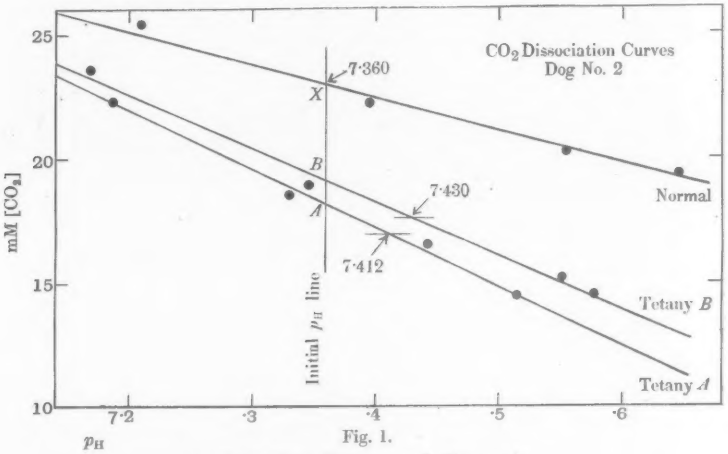

Fig. 1.

A—*X* = Fall in Alkali reserve in Tetany *A*.
B—*X* = " " Tetany *B*.

fall which is maintained throughout the second day, while in the case of number 3 the fall in alkali reserve is delayed until the second day. This change in the alkali reserve is shown in the dissociation curves for the blood of dog number II (Fig. 1) and in the graphs in Figs. 2, 3 and 4. From the graphs it will be seen that in all these cases the fall in H_2CO_3 exceeds that in

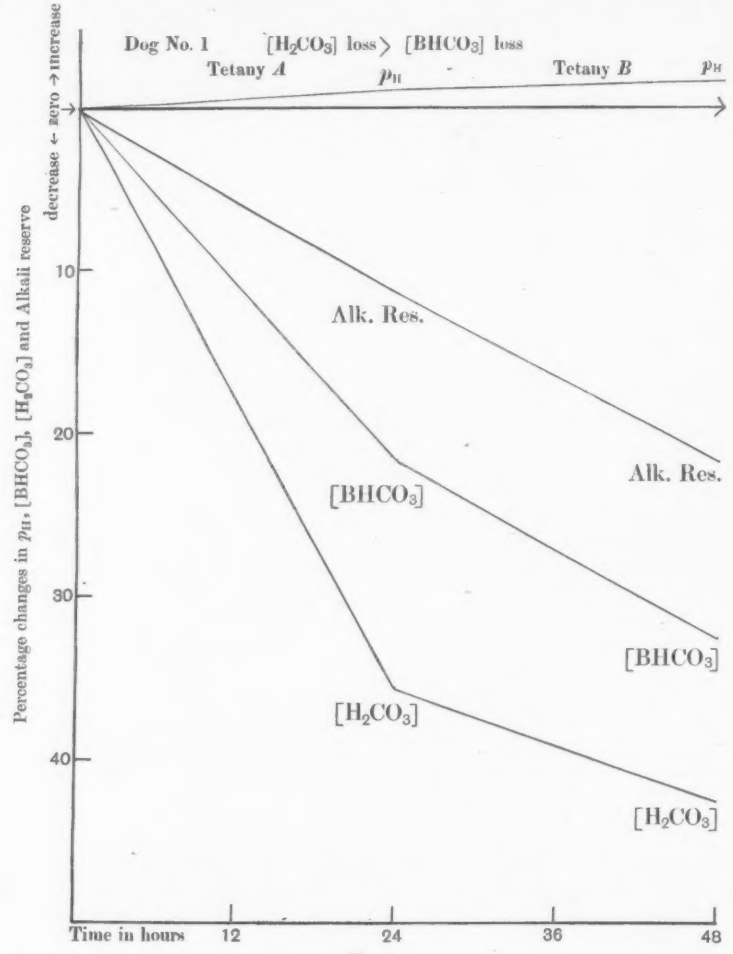

Fig. 2.

$BHCO_3$, thus creating a condition of alkalosis, extending throughout the second day after operation. It is evident from these results that in mild cases of experimental tetany there is a definite though small change in the reaction of the blood, which probably reaches its maximum sometime during the second

day of the disease, and that with this slight change there is a condition of alkalosis, of no great severity it is true, which is occasioned by the fact that there is a greater loss of H_2CO_3 than of $BHCO_3$ (Figs. 2, 3 and 4).

In those cases, however, in which there is a more rapid production of parathyroid tetany and in which the animal shows within 48 hours a definite change from an active, well-conditioned though abnormal individual to one that is manifestly ill and whose *exitus fatalis* is clearly a matter of three or

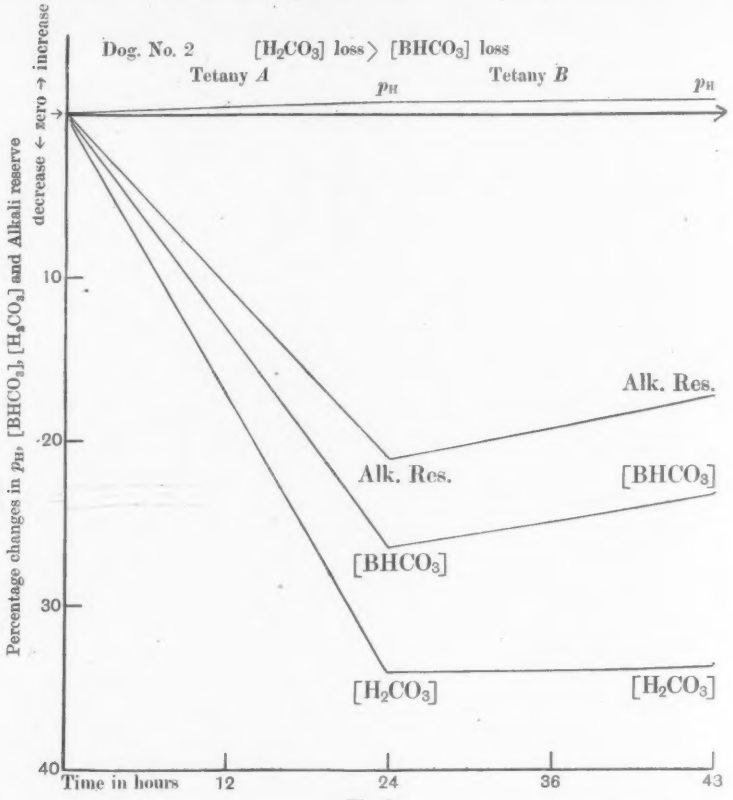

Fig. 3.

four days, the picture obtained is of a type in which one can almost state from the graph the time at which a change from a condition of alkalosis to one of acidosis takes place. An examination of Tables IV, V and VI and of the graphic representation of certain of their details in Figs. 5, 6 and 7, showing the change in alkali reserve on the initial p_H lines and in Figs. 8, 9 and 10, showing the relative percentage changes, will disclose the fact that a primary condition of alkalosis is followed towards the end of the second day by the

opposite condition of acidosis. This swing of the acid-base balance is due to rapid alterations in the H_2CO_3 content. The change in the reaction of the blood is very small, the greatest alteration being 0·163, but small though it is, it would be possible with very accurate and facile methods for p_H estimation to indicate the point in time at which the change from alkalosis to acidosis takes place, namely the point at which the acid and base losses are accurately balanced. In the diagrams, this point is seen to be slightly in advance of the point at which the p_H line crosses the zero or normal line. All of these results indicate consistently that the increase in p_H is small,

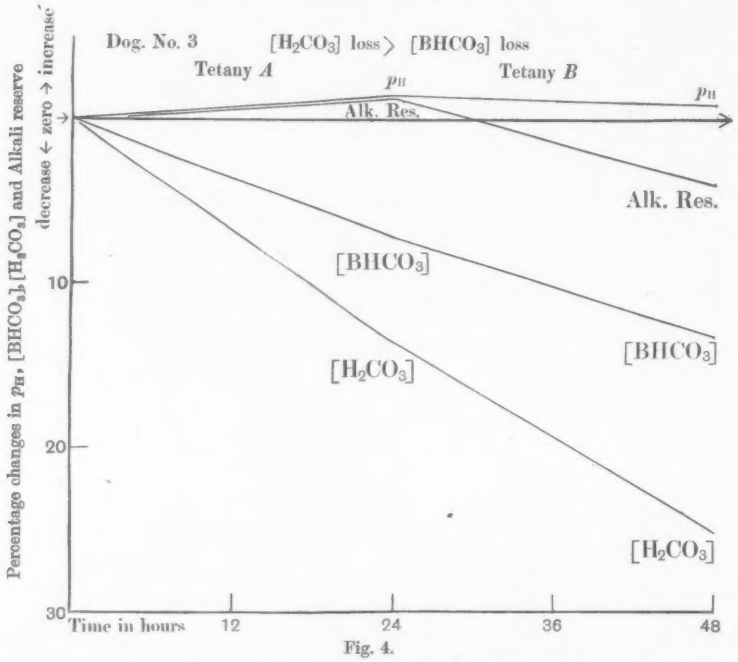

Fig. 4.

that the primary condition is one of alkalosis due to a CO_2 deficit and that this picture is altered to one of acidosis during the development of the signs of acute tetany.

The extreme fall in the alkali reserve in these cases indicates that there is a production of acid or acids other than H_2CO_3. The work of Greenwald [1922] would indicate that lactic acid is produced before muscular tremor is evident and that it is greatly increased when this becomes marked, and further that the increase in lactic acid in the blood is not large enough to account for the total fall in blood alkali. It must be noted that these animals did not show the violent muscular twitchings, with involuntary snapping of the jaws, which

Table IV

Dog No. IV. On the second day after the operation no tremors of the shoulder muscles were visible: slight tremor of legs present with slight spasticity of hind legs. Respiration 70 per minute. Appetite impaired, drinking copiously. Perspiration visible: condition excellent.
On third day after operation, tremor of shoulder, fronto-orbital and leg muscles visible: attempt to move produced coarse spasmodic twitchings of limb muscles. Legs spastic. Respiration rate not increased.

Time in hrs.	Atmosphere % CO_2	Atmosphere p_{CO_2}	Alveolar air CO_2	Blood % CO_2	Blood $[CO_2]$	Blood $[H_2CO_3]$	Blood $[BHCO_3]$	R	p_H	Remarks
0	4·00	28·00	3·685	39·60	17·67	0·840	16·83	18·20	7·44	Normal
24	2·18	15·33	1·975	28·90	12·89	0·460	12·43	26·30	7·603	Tetany *A*
					17·80		16·87			At normal p_H
48	3·62	25·33	3·340	31·49	14·05	0·760	13·29	17·78	7·430	Tetany *B*
					13·70		12·98			At normal p_H
72	3·88	23·66	3·115	25·68	11·45	0·710	10·74	15·14	7·360	Tetany *C*
					9·70		9·20			At normal p_H

Tetany *A*		Total changes	% changes
p_H	=	0·163 +	2·19
p_{CO_2}	=	12·67 −	—
$[BHCO_3]$	=	4·40 −	26·15
$[H_2CO_3]$	=	0·38 −	45·11
Alk. res.	=	0·04 +	0·238

Tetany *B*		Total changes	% changes
p_H	=	0·01 −	0·134
p_{CO_2}	=	3·0 −	—
$[BHCO_3]$	=	3·54 −	21·03
$[H_2CO_3]$	=	0·08 −	9·54
Alk. res.	=	3·85 −	22·90

Tetany *C*		Total changes	% changes
p_H	=	0·08 −	0·608
p_{CO_2}	=	4·34 −	—
$[BHCO_3]$	=	6·09 −	36·20
$[H_2CO_3]$	=	0·13 −	15·48
Alk. res.	=	7·63 −	45·35

Table V

Dog No. V. Marked tetany developed towards the end of the second day. Respiration 240 per minute. Muscle tremor well marked.

Time in hrs.	Atmosphere % CO_2	Atmosphere p_{CO_2}	Alveolar air CO_2	Blood % CO_2	Blood $[CO_2]$	Blood $[H_2CO_3]$	Blood $[BHCO_3]$	R	p_H	Remarks
0	3·910	27·33	3·61	35·95	16·05	0·820	15·13	16·22	7·391	Normal
24	2·647	18·66	2·46	42·62	19·04	0·560	18·48	33·88	7·710	Tetany *A*
					26·40		24·88			At normal p_H
48	3·642	25·67	3·37	31·26	13·95	0·770	13·18	16·98	7·410	Tetany *B*
					14·20		13·38			At normal p_H
72	3·595	25·33	3·33	22·30	9·96	0·760	9·20	12·02	7·260	Tetany *C*
					6·80		6·52			At normal p_H

Tetany *A*		Total changes	% changes
p_H	=	0·319 +	4·32
p_{CO_2}	=	8·67 −	—
$[BHCO_3]$	=	3·35 +	22·15
$[H_2CO_3]$	=	0·26 −	31·7
Alk. res.	=	9·75 +	64·45

Tetany *B*		Total changes	% changes
p_H	=	0·019 +	0·257
p_{CO_2}	=	1·66 −	—
$[BHCO_3]$	=	1·95 −	12·89
$[H_2CO_3]$	=	0·05 −	6·10
Alk. res.	=	1·75 −	11·56

Tetany *C*		Total changes	% changes
p_H	=	0·131 −	1·77
p_{CO_2}	=	2·00 −	—
$[BHCO_3]$	=	5·93 −	39·20
$[H_2CO_3]$	=	0·06 −	7·32
Alk. res.	=	8·71 −	57·50

was so marked a feature in those animals which formed the subjects of the experiments carried out last year. This may be due as mentioned before to the daily withdrawal of blood. Still, in all of these experiments here quoted in detail the animals developed definite tetany and the general uniformity of the results obtained inclines one to the opinion that the first stage of tetany is associated with a condition of alkalosis due to a CO_2 deficit, and that previous to the development of the second phase of the disease, there is a fall in the

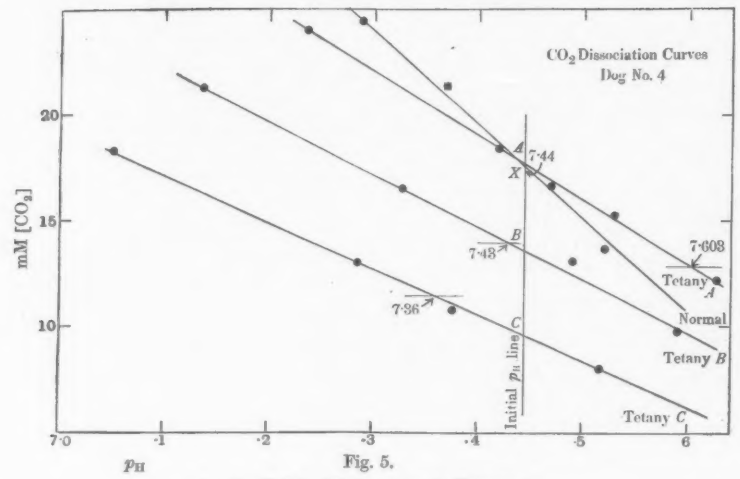

Fig. 5.

A—*X* = Fall in Alkali reserve in Tetany *A*.
B—*X* = ,, ,, Tetany *B*.
C—*X* = ,, ,, Tetany *C*.

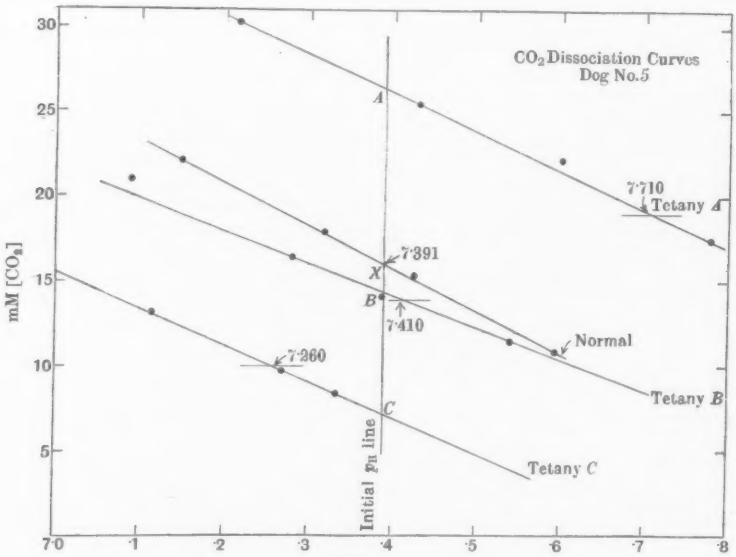

Fig. 6.

A—*X* = Fall in Alkali reserve in Tetany *A*.
B—*X* = ,, ,, Tetany *B*.
C—*X* = ,, ,, Tetany *C*.

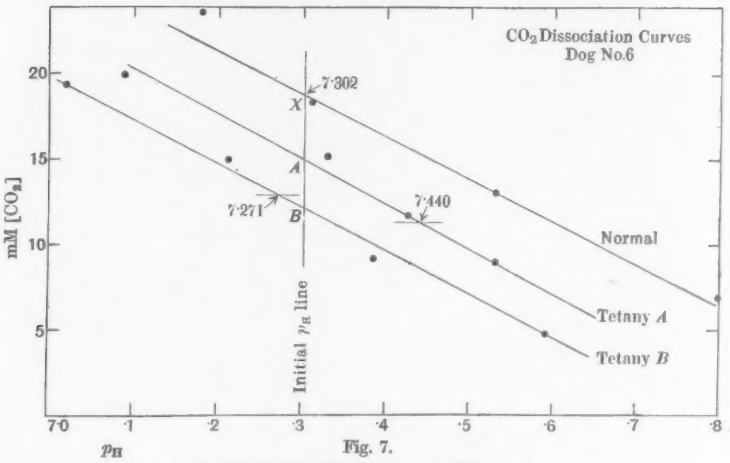

Fig. 7.

A—X = Fall in Alkali reserve in Tetany A.
B—X = „ „ Tetany B.

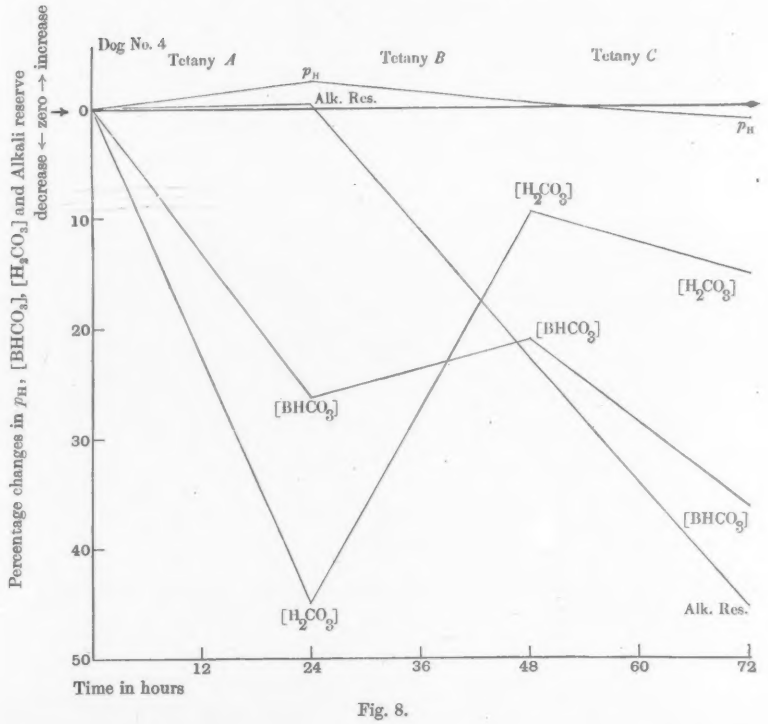

Fig. 8.

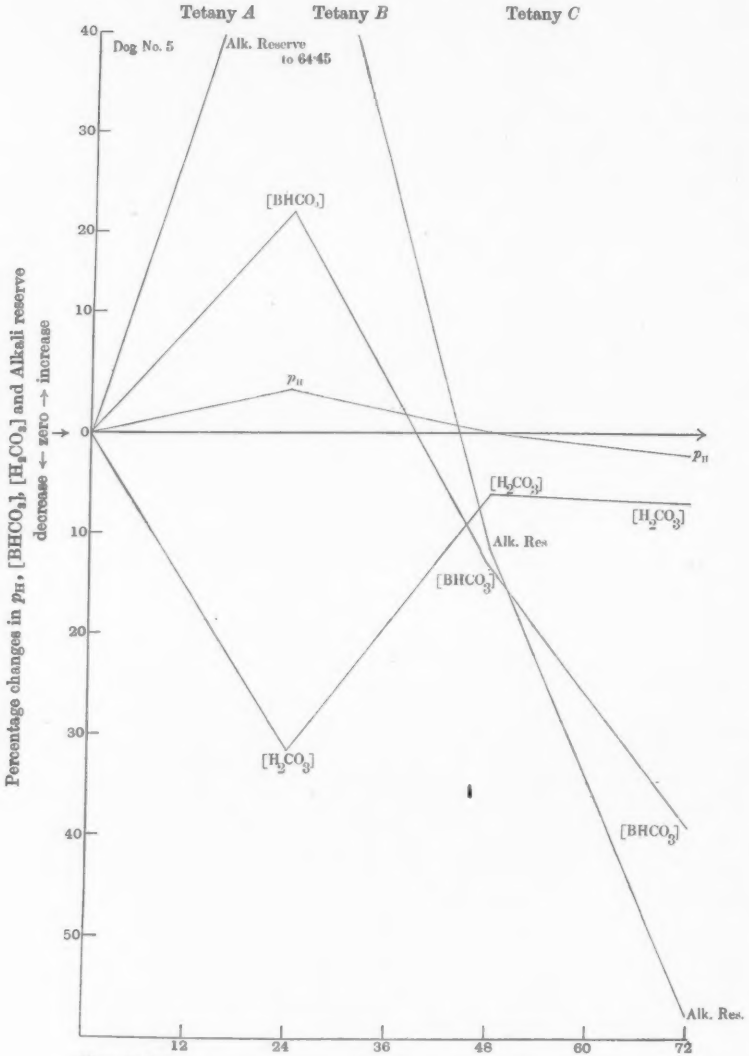
Tetany A
Tetany B
Tetany C
Dog No. 5
Alk. Reserve to 64·45
[BHCO3]
pH
[H2CO3]
Alk. Res
Alk. Res.
Percentage changes in pH, [BHCO3], [H2CO3] and Alkali reserve
decrease ← zero → increase
Time in hours
40
30
20
10
0
10
20
30
40
50
12
24
36
48
60
72

Fig. 9.

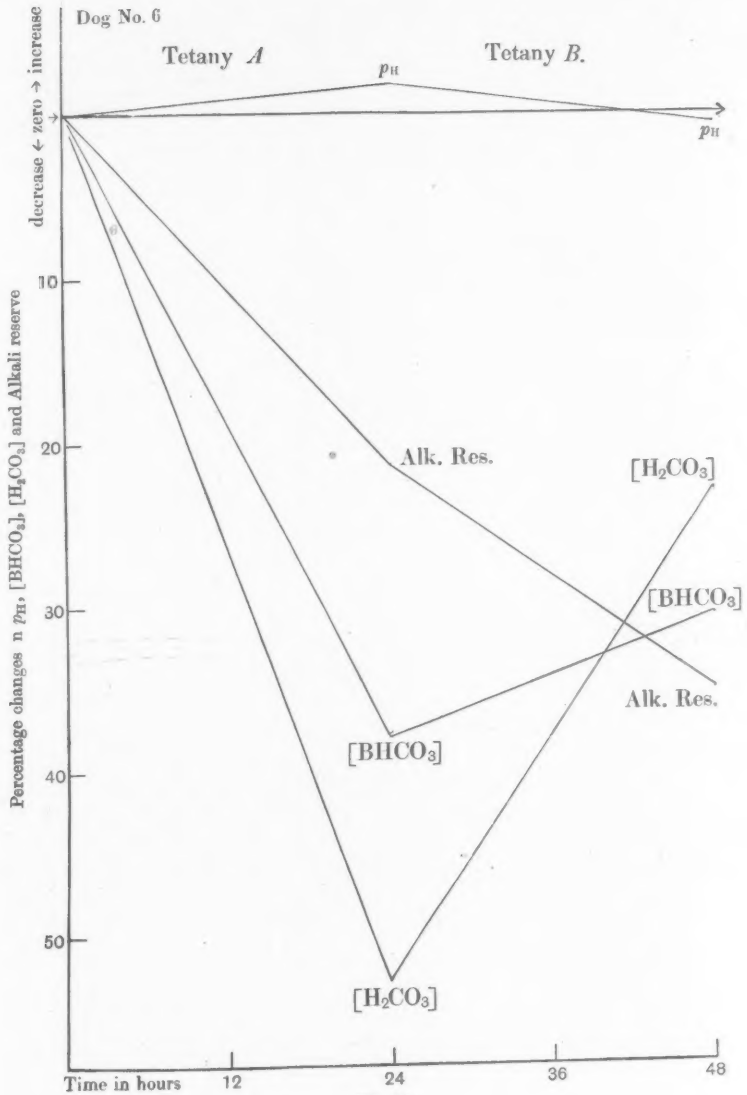
Dog No. 6
Tetany A
Tetany B.
p_H
p_H
decrease ← zero → increase
Percentage changes in p_H, $[BHCO_3]$, $[H_2CO_3]$ and Alkali reserve
10
20
30
40
50
Alk. Res.
$[H_2CO_3]$
$[BHCO_3]$
Alk. Res.
$[BHCO_3]$
$[H_2CO_3]$
Time in hours
12
24
36
48

Fig. 10.

Table VI

Dog No. VI. Definite mild tetany with visible fine muscle tremor. Respiration rate did not exceed 140 per minute.

Time in hrs.	Atmosphere % CO_2	Atmosphere pCO_2	Alveolar air CO_2	Blood % CO_2	Blood $[CO_2]$	$[H_2CO_3]$	$[BHCO_3]$	R	p_H	Remarks
0	5·880	42·50	5·53	41·60	18·57	1·275	17·27	13·54	7·302	Normal
24	2·840	20·00	2·64	25·41	11·35	0·600	10·75	18·20	7·440	Tetany *A*
					14·60		13·60			At normal p_H
48	4·58	32·66	4·30	29·00	12·95	0·98	11·97	13·33	7·271	Tetany *B*
					12·10		11·18			At normal p_H

	Tetany *A* Total changes	Tetany *A* % changes		Tetany *B* Total changes	Tetany *B* % changes
p_H	= 0·138 +	1·89	p_H	= 0·031 −	0·43
p_{CO_2}	= 22·0 −	—	p_{CO_2}	= 9·84 −	—
$[BHCO_3]$	= 6·52 −	37·73	$[BHCO_3]$	= 5·30 −	30·70
$[H_2CO_3]$	= 0·675 −	52·92	$[H_2CO_3]$	= 0·295 −	23·11
Alkali reserve	= 3·67 −	21·25	Alkali reserve	= 6·09 −	35·26

alkali reserve, and a balancing of base and acid, which ultimately results in an increase of acid over base, both being lower than normal, and thus a condition of acidosis is introduced which is steadily increased up to the time of the death of the animal. As to whether or not the alkali reserve of the blood becomes encroached upon during the first 24 hours after operation depends upon the severity and the rate of development of tetany, upon the previous diet of the animal, and also upon the type of the animal. Diet undoubtedly plays a very important part in determining the nature of the subsequent tetany. Here it has been found that all dogs do not develop those signs which are generally regarded as pathognomonic of tetany. It may be that accessory parathyroid tissue is responsible for this. Our experience has been such that we find it possible almost to predict whether the tetany will be accompanied in an active alert dog by violent spasmodic muscular contractions, rapid respiration, profuse perspiration, cardiac irregularity, or in a rather lethargic dog by slight fine tremors, at times difficult to detect, a respiration rate never exceeding 100 per minute and slight perspiration, the dog becoming less and less inclined to move about. The former condition is obtained in short-haired animals of the fox or Irish terrier type; the latter condition, in long-haired animals of the collie type, though usually of very indefinite breed. In all our experiments it has been our constant endeavour to secure animals of the former type.

IV. The hydrogen ion concentration of the blood in tetany.

Reference has already been made to previous work, in which estimations of the p_H of the blood of parathyroidectomised dogs indicated a slight change to both sides of the normal, the average increase being 0·143. The average figure given by Grant and Goldman [1921] for the change in reaction produced by hyperpnoea was 0·160.

Table VII. *The p_H of the blood of normal and thyroparathyroidectomised dogs, determined from CO_2 dissociation curves.*

Dog	Normal p_H	p_H 24 hrs. after operation	Change in p_H	p_H 48 hrs. after operation	Change in p_H	p_H 72 hrs. after operation	Change in p_H
1	7·370	7·590	0·22 +	—	—	—	—
2	7·340	7·420	0·08 +	7·460	0·120 +	—	—
3	7·440	7·603	0·163 +	7·480	0·010 −	7·360	0·080 −
4	7·391	7·710	0·319 +	7·410	0·019 +	7·260	0·131 −
5	7·360	7·420	0·062 +	7·430	0·070 +	—	—
6	7·415	7·520	0·105 +	7·480	0·065 +	—	—
7	7·302	7·440	0·138 +	7·271	0·031 −	—	—
8	7·330	7·480	0·150 +	—	—	—	—
Average	7·369	7·523	0·154 +	7·413	0·044 +	7·310	0·059 −

The range of the change of p_H in the present series with its time relations is shown in Table VII. The average normal p_H obtained was 7·369; the average p_H 24 hours after operation was 7·523, an increase of 0·154; after 48 hours it had fallen to 7·413, an increase above the normal of 0·044; after 72 hours it was 7·301, a decrease of 0·059. It seems justifiable, therefore, to conclude that there is a slight change in the reaction of the blood in parathyroid tetany. The following are average figures from several observers, showing alterations in the p_H of the blood in cases of experimental tetany:

Grant and Goldman [1921]	0·150
Collip and Backus (calculated 1920)	0·240
Cruickshank (colorimetric 1922)	0·146
Cruickshank (dissoc. curve 1923)	0·154

These indicate a rise in p_H, but if the reaction of the blood be followed throughout the period of acute tetany it will be found as shown in the accompanying graphs that it ultimately falls below the normal for the blood under investigation.

V. Alveolar CO_2 tension in tetany.

That hyperpnoea will produce tetany has been demonstrated by Davies, Haldane and Kennaway [1921], Collip and Backus [1920], Bazett and Haldane [1921], and Grant and Goldman [1921]. The forced breathing, which is a sign of parathyroid tetany, causes a reduction in the alveolar CO_2 and by this so-called washing out of CO_2 alters the ratio $BHCO_3/H_2CO_3$ thereby inducing a condition of alkalosis. The theory that the washing out of the CO_2 is a sufficient cause of tetany is not tenable. The causative mechanism, which has as its basis a disturbance of parathyroid function, is more likely to be the nature of a disturbance of the equilibrium of certain ions both in the blood and tissue cells, a disturbance which includes alterations in the stability of the oxyhaemoglobin molecule. It is because of this latter that the tension of O_2 and CO_2 both in alveolar air and in the tissues is of importance. Any mention of the variations of the CO_2 tensions of the alveolar air, without further correlation, can be merely one of interest and it is from that aspect at present that the averages of the CO_2 tensions stated as percentages of dry alveolar air, at 760 mm. Hg., in the various tables are here stated.

Percentage of CO_2 in the alveolar air of normal and tetany dogs.

Normal	Tetany 24 hours	Tetany 48 hours
4·493	2·935	3·49

These figures will indicate, as have the others, that there is a return from a primary phase towards the normal, and it is quite probable that, with the loss of alkali reserve and with the increase in some non-volatile acids or some alterations in the acidity of the haemoglobin molecule, the alveolar CO_2 will increase, keeping pace with the increasing H ion concentration of the circulating blood.

Whatever may be the factors in the production of tetany, the results given in this paper suggest that the essential condition is one of alkalosis only in the first phase of the disturbance and that in fully developed parathyroid tetany the disturbance in the equilibrium of the electrolytes is associated with a reaction of the blood, which is on the acid side of the normal.

Conclusions.

1. The change in the acid-base balance in parathyroid tetany is, in the primary or quiescent stage, such that a condition of alkalosis, due to CO_2 deficit, is produced.

2. During the development of acute tetany, the condition of alkalosis changes to one of acidosis, due to a continuous loss of $BHCO_3$ and an increase in H_2CO_3.

3. Depending upon the rapidity of the development of tetany, the alkali reserve will begin to fall on the first or second day after operation and will continue to fall steadily until the death of the animal.

4. The reaction of the blood, as determined from CO_2 dissociation curves, changes very slightly, the p_H increasing above normal for the animal by an average of 0·154, during the stage of alkalosis, and falling with the introduction of the stage of acidosis.

5. The change towards acidosis is probably hastened but not produced by muscular tremor.

6. The alveolar CO_2 may fall as low as 1·975 % but with the continued loss of base there is a tendency for it to return towards the normal figure.

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IX. A METHOD OF ESTIMATING THE TRYPTOPHAN CONTENT OF CASEINOGEN, BASED ON DETERMINATIONS OF THE NITROGEN VALUES OF THE MERCURIC SULPHATE PRECIPITATE.

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Report to the Medical Research Council.

(*Received October 13th, 1923.*)

In spite of the several attempts that have been made to estimate tryptophan, a glance at the table given below will show that the results achieved hitherto have not been such as to call for much excuse for another attempt. A means of estimating tryptophan is sorely needed, not only in the analysis of proteins, where the figures for this amino acid are still represented by blanks, but also in the analysis of food stuffs connected with a multitude of problems, such as the etiology of pellagra, to mention only a single instance.

The Tryptophan Content of Caseinogen according to various Authors.

Percentage of tryptophan found	According to	Method of estimation
1·5	Hopkins and Cole [1901]	Isolated and weighed
0·5	Abderhalden and Kempe [1907]	Colorimetric (*p*-dimethylaminobenzaldehyde reaction)
0·6	Fasal [1912]	Colorimetric (glyoxylic acid reaction)
0·6	Moraczewski [1913]	From indole produced by bacteria
0·5	Herzfeld [1913]	Colorimetric (*p*-dimethylaminobenzaldehyde reaction)
1·75	Thomas [1914]	Colorimetric (ditto)
1·1	Homer [1915]	Titrated with bromine
1·7	Dakin [1918]	Isolated and weighed
2·0	von Fürth and Nobel [1920]	Colorimetric (Voisenet's formaldehyde reaction)
1·7–1·8	Thomas [1920]	Colorimetric (*p*-dimethylaminobenzaldehyde reaction)
1·54	Folin and Looney [1922]	Colorimetric (phenol reagent)
1·1–1·39	Lüscher [1922]	Colorimetric (benzaldehyde and formaldehyde reaction)
1·50	May and Rose [1922]	Colorimetric (*p*-dimethylaminobenzaldehyde reaction)
2·24	Holm and Greenbank [1923]	Colorimetric (ditto)

It will be seen that the discoverers of tryptophan actually isolated as much of the crystalline amino acid as most workers claim to have estimated by their methods. Dakin, moreover, by extraction with butyl alcohol, obtained

1·7 % at the first attempt, and Onslow [1921] has shown that yields approaching this value may be obtained in routine work.

The colorimetric methods of Thomas, of von Fürth and Nobel and of Holm and Greenbank are the only ones that give values of 1·7 % or greater. As there must be a considerable loss of tryptophan in the mother liquors after crystallisation, it can hardly be doubted that the true value is higher than 2 %, and therefore nearer the figures given in this paper and those of Holm and Greenbank.

Though the colorimetric methods are difficult to carry out, and are liable, according to some of the authors, to an error of 20 %, they are certainly the best yet published. In addition, however, to the low values sometimes obtained, all the usual objections to colorimetric methods may be urged. These objections are especially weighty when the reaction depends not on a single coloured substance, but on two substances of different colours as is the case with tryptophan, *i.e.* "tryptophan blue" and "tryptophan red" [Fearon, 1920]. Any method, therefore, which is based on such familiar procedures as the estimation of nitrogen values, even though open to serious objections, is likely to be an advance in the direction of accuracy.

A description was given in a previous paper [Onslow, 1921] of the compound precipitated by mercuric sulphate from a solution of caseinogen which had been digested by enzymes. Among the amino acids found in addition to tryptophan, there were leucine, cystine, proline, glutaminic and aspartic acids and histidine, but neither lysine nor arginine. The absence of the last two diamino-acids is of considerable importance, as will be seen below.

The work was begun in the hope of obtaining an accurate method for estimating tryptophan. When, therefore, it was found that the total nitrogen of the mercuric sulphate precipitate was no criterion of the tryptophan content, owing to the other amino acids present, an attempt was made to obtain a value for the tryptophan from the difference between the amino nitrogen and the more resistant nitrogen of the pyrrole ring.

Principle of the Method.

In the absence of any diamino-acid, or any heterocyclic acid other than histidine, the total nitrogen of the mercuric precipitate may be considered as belonging to the following fractions, many of which can be estimated:

Total N = Tryptophan N + Histidine N + Monamino N + Peptide N.

It will be seen that account is taken of the fact that a proportion of the amino acids of the mercury compound still remains combined as polypeptides, and, moreover, that proline is only present in an insignificant amount [Onslow, 1921].

In proteins, such as gelatin, which contain very large amounts of proline, special precautions would have to be taken, and, similarly, with proteins containing large amounts of cystine, an estimation of the total sulphur by Benedict's method [1909] would have to be made, so that the correction for

cystine could be applied when using Van Slyke's method for amino nitrogen. From the last equation the amino nitrogen may be expressed as follows:

Amino N = Tryptophan N/2 + Histidine N/3 + Peptide amino N + Monamino N

and hence, the non-amino nitrogen is given by:

Non-amino N = Tryptophan N/2 + Histidine 2N/3 + Peptide non-amino N.

This equation can, of course, be written:

Tryptophan N = 2 [Non-amino N − (Histidine 2N/3 + Peptide non-amino N)].

Tryptophan, it will be seen, is the only unknown term in the equation, because the non-amino nitrogen may be calculated from the difference between the total nitrogen (obtained by Kjeldahl's method) and the amino-nitrogen (obtained by Van Slyke's method); the histidine nitrogen can best be estimated, under these conditions, by the methods of Koessler and Hanke [1919], while the peptide non-amino nitrogen is found from the difference between the amino nitrogen before and after complete hydrolysis. The only term in the last equation not determined is tryptophan, which may therefore be calculated.

The obvious method for completing the hydrolysis of any peptides remaining in the mercuric compound would be to submit them to acid hydrolysis as was done by Levene and Van Slyke [1912] in the case of urine. The great difficulty in doing this is the presence of so large an amount of tryptophan, both free and as polypeptides, which is rapidly destroyed by this treatment, being converted into humin. There is, however, no other method available, because, unfortunately, it has been shown to be impossible to effect complete hydrolysis by means of enzymes[1] [Onslow, 1921; von Fürth and Lieben, 1920; Frankel, 1916], and baryta hydrolysis, the only other possible method, is inapplicable, because the tryptophan, being unsufficiently protected by other amino acids, would break down to indole. Consequently, it is necessary to know the changes undergone by tryptophan during acid hydrolysis. Gortner and his co-workers [Gortner, 1916; Gortner and Blish, 1915; Gortner and Holm, 1917, 1920] have shown that tryptophan and tyrosine are the only two amino-acids[2] concerned in humin formation, and, since tyrosine can be removed from the mercury compound by thoroughly washing, the tryptophan alone is affected. Van Slyke [1912, 1] has shown that the acid hydrolysis of pure tryptophan in no way alters the ratio of the amino nitrogen to the total nitrogen. This important observation was confirmed, and it was also shown, by the following experiment, that when most of the tryptophan is converted to humin by the addition of a carbohydrate, the ratio of total to amino nitrogen remains 2 : 1.

0·25 g. tryptophan and 0·25 g. glucose were boiled in 25 % hydrochloric

[1] This conclusion has been denied by Abderhalden, but Henriques and Gjaldbœk [1911] and Andersen [1915] have challenged this attitude. In any case the method would be useless on account of the large amounts of tryptophan that would be introduced in the erepsin and other enzymes needed.

[2] The data advanced by Roxas [1916] to prove that other amino acids are involved in humin formation, show such low values for these amino acids that they seem rather to support Gortner's contention that if traces of any other amino acids appear to have formed humin, it is by a physical process of adsorption and not by a chemical reaction.

acid for 24 hours on a sand-bath. Distillation with calcium hydroxide in the usual way produced no ammonia. The nitrogen of the humin formed, which amounted to about 79 % of the tryptophan N, was estimated by Kjeldahl, and the filtrate containing the residual tryptophan gave the following figures:

Total nitrogen	6·7 mg.
Amino nitrogen	3·5 mg.

In another experiment, 0·3 g. tryptophan and 0·3 g. glucose were boiled in 25 % hydrochloric acid with the following result:

Total nitrogen	27·7 mg.
Amino nitrogen	13·8 mg.

In this case 25 % of the total nitrogen became melanin. If the ratio of the nitrogen of the residual tryptophan remains undisturbed, the humin must contain equal proportions of nitrogen from the ring, and from the side chain. Thus, if half the melanin nitrogen, formed during complete acid hydrolysis, be added to the amino nitrogen, a correct figure for the total terminal NH_2 groups should be obtained. This, less the original amino nitrogen, should give the non-amino nitrogen bound in the peptides, which is the last term of the equation.

General Scope of Research.

In all, 20 estimations[1] of the percentage of tryptophan in caseinogen were made. During the course of the work, several difficulties were encountered, but most of these were eventually overcome. Of the last eight estimations, six were finally considered to be satisfactory. Four of these, carried out on one sample of caseinogen (English "casein") gave concordant values. The remaining two, carried out on another sample (Merck's "casein") gave concordant, but rather higher, values. Many subsidiary experiments were performed in connexion with the difficulties arising at various points in the course of the estimations.

Outline of Experimental Procedure in Estimation.

A known weight of caseinogen was digested with trypsin in a known volume of fluid. The digest was precipitated with a known volume of the Hopkins and Cole reagent. Free tryptophan, polypeptide of tryptophan, free tyrosine, histidine and probably small quantities of other amino acids were precipitated. The first precipitate was filtered off after four days. The filtrate was allowed to stand. A second precipitate was then deposited and filtered off after 14 days. The two precipitates were washed separately with 7 % sulphuric acid, containing 2–3 % mercuric sulphate, until the wash fluid gave no tyrosine reaction even after standing in contact with the precipitates for 24 hours. The two precipitates were united, suspended in water made slightly alkaline with baryta, and treated with hydrogen sulphide. The treatment was repeated, later in hot

[1] The majority of these were carried out with considerable skill and care by Mr John Lowndes, assistant to the late Mr Onslow. The research owes much to Mr Lowndes' accurate and careful work. (M. W. O.)

solution, until the filtrates from the mercuric sulphide gave no glyoxylic reaction. All the filtrates were added together. The residual mercuric sulphide was then boiled with 10 % baryta solution for an hour and filtered, and the filtrate added to the above combined filtrates. The solution now contained tryptophan, histidine, polypeptide and probably traces of other amino-acids. The barium was quantitatively removed from the solution, which was then reduced *in vacuo* and made up to a known volume. The total nitrogen and amino nitrogen were then determined. The histidine nitrogen was estimated by the method of Koessler and Hanke. Three portions of the original solution were hydrolysed with an equal volume of 6*N* hydrochloric acid in an autoclave for three different periods ranging from 1 to 2 hours at 140°, that is, under a pressure of $3\frac{3}{5}$ atmospheres. In each case the hydrolysed solution was taken down to dryness *in vacuo*. The residue was distilled with calcium hydroxide, thus giving the ammonia nitrogen. The melanin was filtered off, and of this the total nitrogen was determined. The amino nitrogen and total nitrogen were determined in the filtrate. From the above values the tryptophan present can be calculated according to the theoretical equation previously given.

Details of Experimental Procedure.

1. *Caseinogen preparations employed.*

For the first four estimations, English "casein[1]" was used; for estimation V, a small sample of "casein according to Hammarsten" (Merck), and, for estimation VI, English "casein" again. The values of total nitrogen of the solutions given by the decomposition of the mercury precipitates were too low in the first six estimations to give, after allowance for impurities, a value for tryptophan of more than 1·7 %. It was therefore thought possible that racemisation had occurred to some extent in the preparation of the protein used, thus rendering it incapable of digestion and causing the low values.

Hence samples of caseinogen were prepared from milk by two methods. First, the method of Van Slyke and Bosworth [1913] was tried, employing 4 gallons of milk. The product, however, on digestion (estimation VII) did not give a higher value than hitherto obtained for the total nitrogen. Secondly, the method of Mellanby [1915] was tried, and again the product gave no higher value (estimation VIII).

At this stage it became obvious that the cause of low values was to be sought elsewhere. Consequently, for estimations IX to XVIII inclusive "casein according to Hammarsten" (purchased from Messrs Thomson, Skinner and Hamilton) was employed, and during these later estimations, other, hitherto unsuspected, sources of error, affecting the total nitrogen values and consequently the tryptophan values, were discovered. For estimations XIX and XX "casein according to Hammarsten" (Merck) was employed.

[1] 100 % pure casein obtained from Casein Co., Culvert Works, Battersea.

2. *Determination of percentage of water and ash in caseinogen.*

The percentage of water was determined by drying a known weight of caseinogen taken at the time when the digest was started. The drying was carried out in an electric oven heated to 105°. The ash was determined by incineration in a porcelain crucible.

The water content of Hammarsten's casein (T. S. and H.) varied from 8–11 %; the ash varied from 0·7–0·8 %. In the sample of "Hammarsten's casein" (Merck) used, the water content was 10 %, the ash 0·7 %. The actual values for each estimation are given in Table I.

3. *Digestion of caseinogen.*

The caseinogen was digested in a buffer solution, as described in the preparation of tryptophan [Onslow, 1921], in order to prevent the rise of hydrogen ion concentration during hydrolysis. This was found to remain very constant in a solution of $N/10$ sodium acetate, brought to p_H 8·1 by the addition of soda, but it was feared that the tryptophan might be slowly destroyed by oxidases present in the trypsin. When tested for oxidases in the usual way, the sample of trypsin appeared to be practically free. Nevertheless, the following experiment was carried out: 0·5 g. of pure tryptophan was dissolved in 200 cc. of buffer solution. 100 cc. were placed in a round flask *A*, so that a large surface of the fluid was exposed to the air. The remaining 100 cc. were placed in a measuring flask, *B*, so that the fluid just reached to the mark on the narrow neck. The measuring flask was tightly stoppered and fitted with a tube through which hydrogen could be passed. Flask *A* was plugged with wool. A brisk stream of pure hydrogen was passed through flask *B* for 30 minutes, and both flasks placed in the incubator at 37°. The treatment with hydrogen was repeated every two days for seven days, when the tryptophan in both flasks was precipitated as usual, and the nitrogen estimated in duplicate.

Weight of tryptophan taken	Equivalent weight of tryptophan
A. 0·25 g. (aerobic)	0·248 g.
B. 0·25 g. (anaerobic)	0·244 g.
A. 0·25 g. (aerobic)	0·242 g.
B. 0·25 g. (anaerobic)	0·230 g.

From the above figures it appears that no considerable amount of tryptophan is destroyed by oxidation during the process of digestion.

In the next place an active enzyme is necessary, since von Fürth and Lieben [1920] have shown that when caseinogen is digested with trypsin, under the usual conditions, it does not yield more than one-third of the total tryptophan as the free acid. One of the purified dried products is best, since the various extracts prepared from fresh pancreas are too rich in tryptophan and purine bases. Such an enzyme should be strong enough to split off all the tryptophan in a 2 % solution of caseinogen in such a form that it is rapidly precipitated by the mercury reagent of Hopkins and Cole [1901]. Much time was spent in testing

various pancreatic extracts and preparations of trypsin; eventually a solution of Merck's trypsin was used as being most efficient and, at the same time, introducing the minimum quantity of extraneous nitrogen compounds.

The method of procedure was as follows. 50 g. of caseinogen were put into a dry Winchester quart bottle, of accurately known capacity (about 2650 cc.), and about one-third of the bottle was filled with the buffer solution of $N/10$ sodium acetate. The p_H was adjusted by carefully adding strong caustic soda solution until the mixture was alkaline to cresol-red but acid to phenolphthalein.

The enzyme extract was prepared by dissolving 2 g. of Merck's trypsin in 50 cc. of the buffer solution and allowing it to stand in the incubator at 38° for an hour, during which time a small deposit was formed which was filtered off. A solution containing 2 g. of sodium fluoride was also allowed to stand in the incubator and then filtered.

The enzyme extract and fluoride thus prepared were then added to the caseinogen and buffer solution. The reaction immediately became acid, and more soda solution was added until the mixture was faintly alkaline to phenolphthalein. The bottle was then nearly filled with the buffer solution. It is necessary to readjust the reaction continually as, for 24 hours or longer, it is liable to change. When it had ceased to change, the bottle was completely filled with buffer solution. From experience it has been found advisable to keep the reaction at first slightly alkaline to phenolphthalein. If this is done there is less likely to be any undigested deposit at the end of digestion.

The digest was kept in the incubator at a temperature of not less than 38° for 7–10 days. During the last two days, it is possibly advantageous to raise the temperature to 42°.

4. *Precipitation.*

The fluid after digestion was clear, with but a very slight deposit; nevertheless it was always filtered. In the early experiments the filter paper was washed free from tryptophan, and the washings added to the digest. This is undesirable as the washings alter in each case the concentration of the digest for precipitation. Hence, in later experiments, the following plan was adopted, which was quicker and avoided washing. An aliquot portion only of the filtered digest was used, representing 45 g. of caseinogen. Thus the concentration of amino acids and polypeptides in the digest was kept as constant as possible for each precipitation. The filtered digest was made 5 % acid with sulphuric acid, and 150 cc. of the Hopkins and Cole reagent was added for each 1000 cc. of digest. The mixture was allowed to stand for four days, during which time most of the tryptophan and tryptophan polypeptides were precipitated as mercury compounds. It was found, however, that after four days a precipitate continued to be formed which, though consisting mainly of the mercury compound of tyrosine (which deposits more slowly than that of tryptophan), always contained some tryptophan compound. Since the mercury compound

Table I. *Estimation of tryptophan in caseinogen.*

	Number of estimation	Percentage of water in caseinogen	Percentage of ash in caseinogen	Time of digestion in days	Original solution: Total nitrogen mg.	Original solution: Amino nitrogen mg.	Original solution: Non-amino nitrogen mg.	Original solution: Histidine non-amino nitrogen mg.	Hydrolysed portion: Time in autoclave hours	Hydrolysed portion: Amino nitrogen mg.	Hydrolysed portion: Melanin nitrogen mg.	Hydrolysed portion: Ammonia nitrogen mg.	Hydrolysed portion: Total* nitrogen (by addition) mg.	Hydrolysed portion: Total† amino nitrogen (by addition) mg.	Total tryptophan nitrogen mg.	Percentage of tryptophan in caseinogen
English "casein"	XIII	10·9	0·674	7	160·756	53·580	107·176	8·438	1	56·044	30·068	16·130	163·263	76·643	132·06	1·92
									1½	62·839	35·129	18·343	164·212	86·731		
	XV	8·55	0·791	8	160·322	43·862	116·460	9·033	1	61·737	24·365	14·791	159·376	79·022	135·47	1·97
									1½	60·863	30·220	16·639	158·450	81·713		
									1½	59·980	35·458	18·489	159·376	84·088		
	XVI	8·55	0·791	25	167·718	51·950	115·768	20·301	1	59·424	22·516	21·570	168·004	77·131	134·84	1·97
									1½	61·035	24·981	23·111	—	80·435		
									1½	59·466	27·755	24·035	—	80·529		
	XVIII	8·0	0·673	27	177·742	55·628	122·114	28·251	1½	59·105	26·670	27·567	—	80·793	128·37	1·87
									1½	62·243	32·489	28·486	—	87·118		
									1½	58·615	34·021	28·792	—	84·349		
Merck's "casein"	XIX	10·0	0·667	14	187·954	56·581	131·373	25·415	1 1/3	60·667	28·202	15·186	187·462	79·278	147·69	2·15
									1½	69·352	30·707	15·655	187·305	89·354		
									1½	65·949	36·656	15·968	187·931	89·019		
	XX	10·0	0·667	14	174·804	62·640	112·164	16·057	1½	64·807	27·263	7·828	174·782	80·904	146·51	2·14
									1¾	66·088	32·585	12·524	174·780	86·325		
									2	62·889	36·030	15·029	173·216	85·638		

All figures for the original solution are calculated on 50 g. of dry caseinogen.

* Includes the total nitrogen of the filtrate from the melanin together with the ammonia and melanin nitrogen.

† Includes the amino nitrogen of the filtrate from the melanin together with that of half the melanin and of the calculated proportion of ammonia.

Table II. *Shows the distribution of nitrogen after hydrolysing mixtures of tryptophan and histidine in an autoclave.*

	Percentage of histidine nitrogen of total nitrogen	Total nitrogen calculated mg.	Total nitrogen found mg.	Amino nitrogen calculated mg.	Amino nitrogen found mg.	Portion hydrolysed in autoclave: Time in autoclave hours	Amino nitrogen mg.	Melanin nitrogen mg.	Ammonia nitrogen mg.	Total nitrogen (by addition) mg.	Deficiency of amino nitrogen	Percentage of total ammonia nitrogen derived from amino nitrogen
Exp. I	13	125·800	126·990	—	—	1	—	3·315	2·536	126·102	—	—
,, II	10·5	153·240	155·286	73·950	73·971	4	—	11·177	10·145	148·674	—	—
,, III (*a*)	18	167·275	167·460	78·625	79·460	4	70·146	12·445	10·398	—	3·092	29·7
(*b*)	—	—	—	—	—	6	—	4·837	3·551	166·174	—	—
(*c*)	—	—	—	—	—	18	65·021	19·039	16·485	166·934	4·920	29·9
,, IV	8	149·230	—	72·610	—	24	62·332	18·278	3·297	149·289	1·139	34·5
,, V	24	180·307	—	82·969	—	24	70·615	21·322	5·580	179·616	1·693	30·3
,, VI	0	137·200	137·026	68·600	68·556	24	61·278	12·952	1·775	137·007	0·846	47·7

of tyrosine has subsequently to be removed by washing, and this is a slow process, the second precipitate was filtered off separately, so as to avoid contaminating the first precipitate with more tyrosine compound than necessary. After 14 days a precipitate still continued to form, but this rarely contained tryptophan to any appreciable extent. If, however, the digestion is either not good or not sufficiently long in duration or both, the precipitate may contain tryptophan even after 16 days' deposit. It is possible that, in these circumstances, polypeptides of tryptophan were present to a greater extent, and were but slowly precipitated. But, for a good digest of 10 days' duration or more, 18 days should suffice for total precipitation of the tryptophan compounds.

5. *Washing to free precipitates from tyrosine compound.*

The two mercury precipitates were filtered on hardened folded papers on an ordinary funnel. They were then washed with a wash-fluid containing 2–3 % mercuric sulphate in 7 % sulphuric acid. In the majority of estimations, I–XII, the precipitates were washed with 7 % sulphuric acid alone, and, as the washings gave for some time no glyoxylic reaction, it was assumed that the 7 % acid only removed the mercury tyrosine compound. This is true at first; but, after prolonged washing, when the tyrosine compound is almost completely removed, it was found by accident, on testing the wash fluid again, that tryptophan was present and was being removed from the precipitate, probably owing to final removal of mercury by the 7 % acid. This loss vitiated the results of estimations I–XI. For the later estimations, the wash-fluid always contained mercuric sulphate, and the washings were repeatedly tested with glyoxylic reagent at every stage. There was thus no loss of tryptophan during the process.

The washings were tested for tyrosine with Millon's reagent, and washing was continued until this test was negative. It was found, towards the end of the washing, that if the wash fluid was allowed to run through the precipitates, it would give a negative tyrosine reaction, but if the fluid was allowed to stand on the precipitate by blocking the neck of the funnel, after 24 hours a strong tyrosine reaction might be given. Hence, towards the end of the washing operations, the fluid was always allowed to remain in contact with the precipitates for 24 hours or longer until it gave a negative Millon reaction.

The first precipitate was free from tyrosine in about three weeks, but the second precipitate, though containing but little tryptophan, needed six weeks' washing or more. If, however, the digests were set up in advance, the later stages of estimation could be carried out while the preparation of further precipitates was in progress. The amount of tryptophan in the second precipitate was so small, that it was thought inadvisable to attempt to re-precipitate it with mercury reagent with a view to removing the tyrosine compound in this way.

6. *Decomposition of mercury precipitate with hydrogen sulphide.*

After tyrosine was completely removed, the precipitates were washed once or twice with water. They were then washed off the papers (these being reserved) and suspended in water which was made slightly alkaline with baryta. Hydrogen sulphide was then passed in for a considerable time, and the liquid filtered. This process was repeated several times. The filter-papers also were suspended and treated separately. Finally, the papers and mercuric sulphide were suspended in water, heated on a water-bath, and again hydrogen sulphide passed in. This was repeated until the filtrate and washings from the sulphide gave no glyoxylic reaction. The various filtrates and washings were then all united. For estimations I–XII, the treatment of the sulphide precipitate ceased at this stage. Even, however, after the precautions mentioned in sections 4 and 5 above as regards precipitation and washing had been taken, the value obtained for tryptophan content, on completing the estimation, was only about 1·7 % as a rule. As mentioned in the Introduction, 1·7 % of crystalline tryptophan has been obtained by Dakin from a caseinogen digest[1]; therefore the total content should be higher. It was difficult to imagine where any loss of tryptophan had taken place, but the fact that the mercuric sulphide was found to contain nitrogen, even after well washing with hot water and with alcohol, led to the suggestion that some tryptophan might be retained in the sulphide, either by adsorption or from some other cause, such as incomplete decomposition. The mercury sulphide residue from one estimation, therefore, after the final washing with hot water, was boiled with 10 % baryta for one hour as a method of extracting any residual tryptophan. The barium was quantitatively removed from the filtrate from the sulphide, and the solution tested for tryptophan with glyoxylic reagent. Quite a good reaction was given. Hence in all subsequent estimations, the mercuric sulphide was always extracted with 10 % baryta, and the extract, after removal of barium, was added to the original filtrate from the hydrogen sulphide decomposition.

7. *Reduction* in vacuo.

The solution obtained from 6 was quantitatively freed from barium with dilute sulphuric acid; this left the solution slightly acid owing to the hydrogen sulphide. The barium sulphate was filtered off, and the filtrate concentrated *in vacuo* on a water-bath. As the hydrogen sulphide passed off, the solution became slightly alkaline; hence there should be no danger of the tryptophan being altered by heating on the water-bath. After concentrating the main bulk, the last washings of the barium sulphate precipitate may be concentrated separately and added later, in order to avoid heating the main bulk of tryptophan solution longer than is absolutely necessary. The reduction *in vacuo* was carried sufficiently far to enable the residue and washings to be made up to a volume of 100 cc. Henceforth, this solution will be referred to as the original solution.

[1] Due, no doubt, to the fact that fresh extract of pancreas was used, which is impossible, as already explained, in the present procedure.

8. *Determination of total and amino nitrogen.*

The total nitrogen of 5 cc. (in duplicate) of the original solution was then estimated (Kjeldahl), incineration being carried on for three-quarters of an hour after the mixture was clear. In the majority of estimations I–XII, this figure was too low, as mentioned above; for, when the value of the histidine present was subtracted, the remaining figure, even if it represented tryptophan entirely (which was unlikely owing to presence of traces of other amino acids), would only give a value at most of 1·7 % or, more frequently, of 1·3–1·5 %. After the method of extraction of the mercuric sulphide with 10 % baryta solution was adopted, the total nitrogen value was raised; it still varied considerably, however, as was to be expected, since the concentration of polypeptides and histidine in each digest varied and, consequently, different amounts of these substances were precipitated.

The total amino nitrogen was estimated on 1 cc. of the original solution (Van Slyke). This value also varied in each estimation for the same reasons as caused variation of the total nitrogen.

9. *Determination of histidine.*

Several methods have been described for this purpose, and much time was spent in testing them. Lautenschlager's [1918] titanium method was given an exhaustive trial, but theoretical results could not be obtained, even when the titanium procedure was replaced by Limpricht's [1878] method of reduction with stannous chloride (technical method still employed in Germany). There can be no doubt that under the given condition, diazobenzenesulphonic acid does not react quantitatively. According to Lautenschlager, one molecule of the diazo reagent reacts with one molecule of titanium and of histidine; according to Meyer [1909], four molecules of titanium react with each azo-group, and, according to Pauly [1915], the histidine compound is a bi-azo compound, which decomposes, yielding 20 % of its nitrogen when heated in either acid or alkaline media. The work of Weisz and Ssobolew [1914] shows that the concentration of histidine has a powerful influence on the production of the bi-body, and the observations of Hanke and Koessler [1920] show that the temperature at which, and the order in which, the reagents are added have a further influence.

The copper method of Kober and Sugiura [1913] was also tried. It was found to be very accurate for some of the amino-acids, but the observation of these authors that amino-caproic acid (*nor*-leucine) quantitatively precipitates all the copper except that of histidine, could not be verified, at least in mixtures containing the products of hydrolysis.

Finally, the colorimetric method of Koessler and Hanke was adopted as preferable to that of Weisz and Ssobolew. It is convenient for very small amounts of histidine, and even if not very accurate, the error introduced cannot be very great.

The procedure was as follows. To 5 cc. of the original solution in a small glass basin, 2·25 cc. of concentrated hydrochloric acid were added and the mixture made up to 12·5 cc. This was heated in the basin on a water-bath, and 2·5 cc. of 15 % phosphotungstic acid solution added, which precipitates the histidine both free and as polypeptide. The solution was immediately taken off the bath, stirred well and allowed to stand on ice for four days. The histidine precipitate was then filtered off, washed with fluid consisting of 15 g. of phosphotungstic acid, 18 cc. of concentrated hydrochloric acid and 200 cc. of water saturated with histidine phosphotungstate. The histidine precipitate was dissolved in the smallest quantity of 10 % caustic soda and made up to 250 cc. This solution was then used for estimation.

The method of Koessler and Hanke is based on the colour reaction given by histidine with *p*-diazobenzenesulphonic acid. This was compared colorimetrically with a standard indicator solution.

The reagent was prepared as follows: 4·5 g. of sulphanilic acid were dissolved in 45 cc. of 37 % hydrochloric acid (sp. gr. 1·19) and made up to 500 cc. 25 g. of 90 % sodium nitrite were dissolved in water and diluted to 500 cc. Then 1·5 cc. each of the sulphanilic acid and sodium nitrite solutions were measured into a 50 cc. volumetric flask. The flask was then immersed in an ice-bath for five minutes. Then 6 cc. more of the sodium nitrite solution were added, and the well-mixed solution again allowed to lie in the ice-bath for five minutes. The mixture was made up with distilled water to 50 cc., and the flask returned to the ice-bath where it was kept. This reagent must not be used for at least 15 minutes after diluting with water. It is best to prepare a fresh reagent every day.

The standard solutions for the indicator were prepared as follows: 0·5 g. of Grübler's methyl orange was dissolved in water and diluted to 500 cc.: 2·5 g. of Grübler's Congo red were mixed with 50 cc. of absolute alcohol and made up to 500 cc. with water.

For the estimation of histidine, 1 cc. of the Congo red and 1·1 cc. of the methyl orange were allowed to flow into 250 cc. of water contained in a 500 cc. volumetric flask. It was then made up with water. The indicator solutions must not be mixed in concentrated form.

The estimation now proceeded as follows: $(l–x)$ cc. of water and 5 cc. of a 1·1 % sodium carbonate (Merck) solution were measured into the left-hand cylinder of a Kober colorimeter. Then 2 cc. of the reagent were measured into a 5 seconds delivery 2 cc. pipette, the time noted to the second, and the reagent allowed to flow into the alkali. The contents of the cylinder were then thoroughly mixed. Finally, x cc. of the histidine solution were allowed to flow into the cylinder exactly 1 minute after the reagent began to mix with the alkali. The contents of the cylinder were again thoroughly mixed, the cylinder transferred to the colorimeter and the instrument set at 20 mm. 8 cc. of the indicator solution were put in the right hand cylinder which was then adjusted to match.

Before estimations can be made, a curve must be plotted using a solution of known strength of histidine hydrochloride. The number of mm. on the scale of the colorimeter can then be read off as values of histidine.

It was found advisable to take readings about every minute for half an hour in order to record the maximum development of colour. Also the tube of the colorimeter should be repeatedly screwed up and down in the cup in order to secure thorough mixing. The values read on the right-hand scale should not exceed 20 mm.

10. *Hydrolysis in the autoclave and determination of amino, melanin and ammonia nitrogen.*

The next question to be considered was the length of time of hydrolysis necessary to decompose the peptides completely. Van Slyke [1912, 2] in his study of this subject, has shown that one and a half hours in an autoclave at 150° will complete the hydrolysis of caseinogen and most other proteins. Owing to the very different composition of the present mixture, it seemed necessary to repeat this work, and as the figures show (Table I), complete hydrolysis, as defined by the maximum figure for the amino nitrogen, was certainly obtained at the end of one and a half hours at 140°, if not earlier.

The method of procedure was as follows. Amounts, each of 20 cc., of the original solution were heated in a flask with an equal volume of 6*N* hydrochloric acid, for three periods ranging between one and two hours, in an autoclave at 140°. The hydrolysed mixture was then distilled *in vacuo* on a water-bath to dryness, whereby the hydrochloric acid was removed. The residue in the flask was washed out into a Claisen flask, made alkaline with 15 cc. of a 10 % suspension of calcium hydroxide, 100 cc. of alcohol were added and the mixture distilled at a temperature below 45° for half an hour into a known volume of standard alkali. (It is important to wash out into the Claisen flask, otherwise fumes of hydrochloric acid may not be completely removed.) In this way any ammonia present was determined. The melanin was filtered off from the residue in the flask and carefully washed until free from chlorides. The total nitrogen of the melanin on the filter paper was then estimated (Kjeldahl). The filtrate was concentrated *in vacuo*, and the volume made up to 50 cc.; of this the total nitrogen and the amino nitrogen were then determined (Kjeldahl and Van Slyke).

Values for Tryptophan Content obtained.

The figures for six of the last eight estimations are given in Table I. The first twelve estimations, though affording experience in the method, were vitiated by the errors mentioned in sub-sections 4, 5 and 6. Two of the last eight estimations were discarded on account of a suspected loss due to incomplete precipitation. A point to be noted is that the amount of histidine precipitated appears to increase with the time of digestion. This is probably due to the fact

that more free histidine is present, and hence the concentration is greater and more is precipitated.

The greatest difficulty in the interpretation of the results is offered by the production of ammonia during hydrolysis in the autoclave. The important point at issue is whether any, and, if so, how much of the ammonia arises from the amino groups. Apart from general interest, its origin is irrelevant, except in so far as it affects the total amino nitrogen value (made up of the amino value of the hydrolysed solution together with half the total nitrogen of the melanin). The whole question is considered in detail in the next section, where the results of a separate research on the origin of ammonia will be given, and it will be shown how the proportion of ammonia arising from the amino groups was determined for each estimation.

The next point to be considered is the total amino nitrogen after hydrolysis in the autoclave, for it is this figure that is essential in solving the equation (p. 65).

Fig. 1, constructed from the values obtained in estimation XX, represents the distribution of nitrogen during hydrolysis: this is also confirmed, in essential, by some preliminary experiments on hydrolysis for different periods and also by graphs constructed from the values obtained in ten, at least, of the other estimations—in fact in all where such values were determined.

From Fig. 1 it will be seen that during hydrolysis the amino value of the filtrate from the melanin, at first increases, but is eventually falling at the end of two hours. On the other hand, both the melanin and ammonia continue to increase. Since the ratio of total to amino nitrogen in the melanin remains unaltered, it will be seen that half the melanin value at any point should be added to the amino values. Further, it will be shown on p. 81, as we have said, that a certain proportion of the ammonia is also derived from the amino nitrogen. Hence the true amino value at any point is the sum of the amino nitrogen estimated, together with half the melanin value and a certain proportion of the ammonia value. Moreover, the total amino value (as calculated above) should remain constant, once complete hydrolysis has taken place; that is, the falling amino value (estimated) should always be compensated by the due proportions of melanin and ammonia, provided, as there is reason to believe, there is no other source of loss of nitrogen. This final value, therefore, is a measure of the total amino nitrogen of the original solution after hydrolysis, and has been used in solving the equation in every estimation.

Since the amount of polypeptide in the original solution may always vary, it will be seen that separate hydrolyses are essential in the case of each estimation. For the amount of caseinogen employed in these estimations, it is not possible to conduct more than three hydrolyses in the autoclave in each estimation. Several preliminary trials, however, were made, before the estimations were commenced, of hydrolysis in the autoclave for periods between $\frac{1}{2}$–$2\frac{1}{2}$ hours, and the values all indicated the same course of events as is recorded in the accompanying figure.

In estimations XIII and XV, unfortunately, the period of one and a half hours was not tried. Hence the value at one and a quarter hours has been taken, but there is little doubt that this is the maximum when the remaining estimations are considered, for in these the amino values at one and a quarter and one and a half hours are practically identical. In estimation XVIII, it

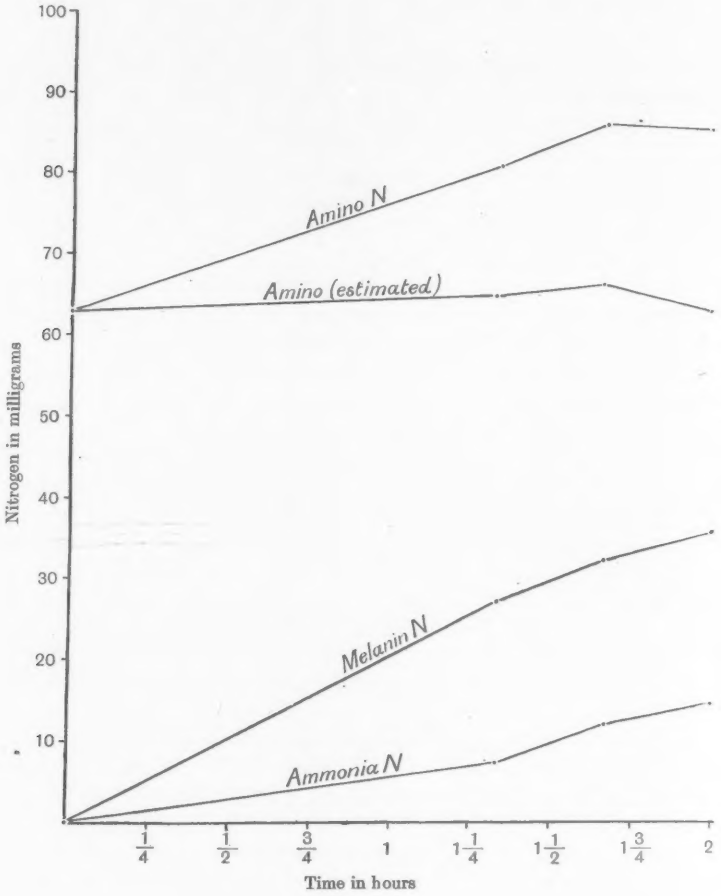

Fig. 1.

is true, the amino value at one and a third hours is too high, owing to the melanin value being too great as is seen on plotting a graph. For estimations XVI–XX inclusive, therefore, the mean of the amino values at one and a quarter and one and a half hours has been taken as the final value.

As the result of employing the values in Table I for solving the theoretical equation, the percentage of tryptophan in 100 g. of dry caseinogen (corrected for ash) is as follows:

English "casein"	Merck's "casein"
%	%
1·92	2·15
1·97	2·14
1·97	
1·87	

Sources of Error in Estimations.

The possible sources of error in the values given above must now be considered. The chief possibility of error lies, no doubt, in the origin of ammonia formed in hydrolysis. Other, very slight, errors, as it will be shown, may be introduced by the enzyme extract employed and by the presence of proline. All these will be dealt with under the following sub-sections.

1. *Production of ammonia during hydrolysis in the autoclave.*

To the problem as to the origin of ammonia much time and experiment were given. The suggestions made and experiments performed are given under the following headings:

(*a*) That the ammonia is derived from amide nitrogen present in the original solution. Such nitrogen is said to be driven off by heating with 20 % hydrochloric acid for one hour on a water-bath, and then estimating as ammonia in the usual way. No amide nitrogen was obtained when the original solution from pure caseinogen was used.

(*b*) That the ammonia is derived from tryptophan. 100 mg. of tryptophan were heated with an equal quantity of 6*N* hydrochloric acid in an autoclave at 140° for two hours. 1·13 mg. of melanin nitrogen were found, but no ammonia. A mixture of tryptophan and glucose was treated similarly; 10·1 mg. of melanin were found but only a very small amount of ammonia nitrogen (0·5 mg.). After hydrolysis, the amino nitrogen was found to be 50 % of the total, as should be expected on the hypothesis that the ratio in the melanin remains unchanged.

(*c*) That the ammonia is derived from tryptophan in the presence of small quantities of other amino acids such as might occur in the original solution. Hence tryptophan (200 mg.), alanine (10 mg.), aspartic acid (15 mg.), cystine (5 mg.), glutaminic acid (15 mg.), glycine (10 mg.), leucine (30 mg.), tyrosine (5 mg.) and phenylalanine (10 mg.), together with lactose (10 mg.) were hydrolysed as usual in an autoclave for two hours; 0·7 mg. of ammonia nitrogen was found.

(*d*) That the ammonia is derived from purine introduced by trypsin. 100 mg. of uric acid, heated in the autoclave as usual for two hours, gave 1·41 mg. of ammonia nitrogen. It will be seen, however, on p. 83 that the amount of purine nitrogen introduced with the trypsin solution could only provide a very small amount of ammonia.

(*e*) That some substance precipitated with the mercury precipitate catalyses the production of ammonia. 100 mg. of tryptophan together with 3 cc. of the original solution were hydrolysed as usual for one and a half hours. 0·01 mg. of ammonia nitrogen was found.

(*f*) That the presence of polypeptide leads to production of ammonia. A solution of peptone, polypeptides and amino acids (alcoholic extract of Witte's peptone) was hydrolysed in the autoclave as usual for one and a half hours. 6·64 mg. of ammonia nitrogen were found.

(*g*) That tryptophan, in the presence of other amino acids, is catalysed by some substance in the original solution. A mixture of tryptophan and amino acids similar to those employed in (*c*), together with 5 cc. of the original solution, was heated in the autoclave as usual for one and a quarter hours. 0·25 mg. of ammonia nitrogen was found.

(*h*) That the presence of polypeptide affects the production of ammonia from tryptophan. A mixture of tryptophan (100 mg.), alanyl-glycyl-glycine (300 mg.) and lactose (10 mg.) was heated in the autoclave as usual for two hours. 0·28 mg. of ammonia nitrogen was found.

Except in the case of the polypeptide mixture in (*f*), which contained substances other than are present in the original solution, none of the experiments (*a*) to (*h*) offers a solution as to the source of ammonia.

Hence this remained uncertain, until a number of additional estimations had been made: from these it appeared that the amount of ammonia produced bears a relation to the amount of histidine present. This is illustrated by the following figures taken from graphs constructed from values given in Table I.

No. of estimation	Percentage of histidine N of total N	Ammonia mg. formed during hydrolysis in autoclave	
		During 1 hour	During 1½ hours
XIII	8	15	23
XV	8	15	22
XVI	18	19	28
XVIII	24	22	32
XX	17	7	11
XIX	20	12	18

In the early stages of the work, the amount of histidine present had been thought too small to account for the ammonia produced. However, the presence of sufficient histidine in the majority of the estimations, coupled with the above relationships, led to the suggestion that much of the ammonia might be produced from the histidine.

Hence solutions of pure tryptophan and pure histidine monohydrochloride were made up, in which the total nitrogen was of a value comparable to that found in the original solution from the digest, and in which the histidine nitrogen was present to the extent of 8, 10, 13, 18 and 24 %; in one solution tryptophan alone was employed. Aliquot portions of these solutions were heated with an equal volume of 6*N* hydrochloric acid in the autoclave for various periods at the usual temperature and pressure. The ammonia, melanin,

amino and total nitrogen values were determined in the usual way. The results are seen in Table II.

Certain deductions can be made from the above data. First, that ammonia is, without doubt, formed from both tryptophan and histidine, but in greater quantity from the latter under the above conditions. Secondly, that the rate of formation of melanin (and probably ammonia) is catalysed by some substance present in the original solution from the digest, which is absent from the pure amino acids, tryptophan and histidine. In all the above experiments, much less ammonia and melanin were formed than from the original solution, although the length of time of hydrolysis was much greater. It could also be seen that melanisation took place very slowly, for in Exps. III (*c*), IV and V, the hydrolysed solution was taken out of the autoclave and examined at intervals of about four hours. Examination showed that, unlike what occurs in the original solution in which 20–30 mg. of melanin are formed in the first hour, as a rule very little melanin was formed during the first four hours, and the amount gradually increased with the time of hydrolysis.

That a very small influence may greatly affect the rate of melanisation is also illustrated in the above experiments; for in Exps. I, II and III (*a*), one sample of hydrochloric acid was used, for Exps. III (*b*), (*c*), IV and V another sample. Thus, in spite of the fact that the tryptophan solution used in Exps. III (*a*), (*b*) and (*c*) was identical, the two different samples of acid (which were initially of the same strength and apparently equally pure) brought about melanisation at different rates. A similar phenomenon is illustrated in Exps IV and V, as contrasted with III (*c*), for in IV and V, with the same acid, a sample of tryptophan was used which was more pure (as determined by the total and amino nitrogen, see Table II) than that employed in Exps. I, II and III. Consequently in IV and V, melanisation was slower.

The above results make all the preliminary hydrolyses of various mixtures of amino acids (*a*) to (*f*) unsatisfactory, for it is obvious that the pure amino acids should be subjected to many hours' hydrolysis in the autoclave before any deductions can be drawn as to formation of ammonia.

The conclusions finally to be drawn are that, in the solution from the digest, some substance catalyses melanisation and, very probably, the formation of ammonia; and that histidine contributes largely to the ammonia.

The main question at issue with regard to the ammonia in the estimation of tryptophan is, however, the determination of what proportion of it arises from deaminisation. For we are concerned with the ammonia nitrogen, only in so far as it affects the total amino value after hydrolysis.

The difficulty experienced in obtaining a correct estimation of the proportion of ammonia derived through deaminisation in the original solution from the digest is brought about by the possible presence of traces of aliphatic amino acids. Since, however, the latter can only be present in relatively very small amounts, the greater part of the ammonia no doubt arises from the histidine and to some extent from the tryptophan. The only course, therefore,

to adopt is to take a percentage of the ammonia, formed on hydrolyses of the original mixture, based on that formed by deaminisation in the mixtures used above in Exps. III, IV and V. This has been obtained as follows.

By reference to Table II, it will be seen that even when half the value of the melanin nitrogen in each case is added to the amino value determined after hydrolysis, there is a deficit in the amino value as compared with that originally determined before hydrolysis. The deficit of nitrogen, therefore, represents the proportion of ammonia nitrogen arising from deaminisation. Expressed as a percentage of the total ammonia it is for four experiments as follows:

	Percentage of histidine N of total N	Percentage of total ammonia N derived from amino N
Exp. III (*a*)	18	29·7
„ III (*c*)	18	29·9
„ IV	8	34·5
„ V	24	30·3
„ VI	0	47·7

From the above values Fig. 2 has been plotted, thus enabling one to obtain the proportion, approximately, of ammonia derived from the amino groups for any percentage of histidine within the range of the actual estimations made.

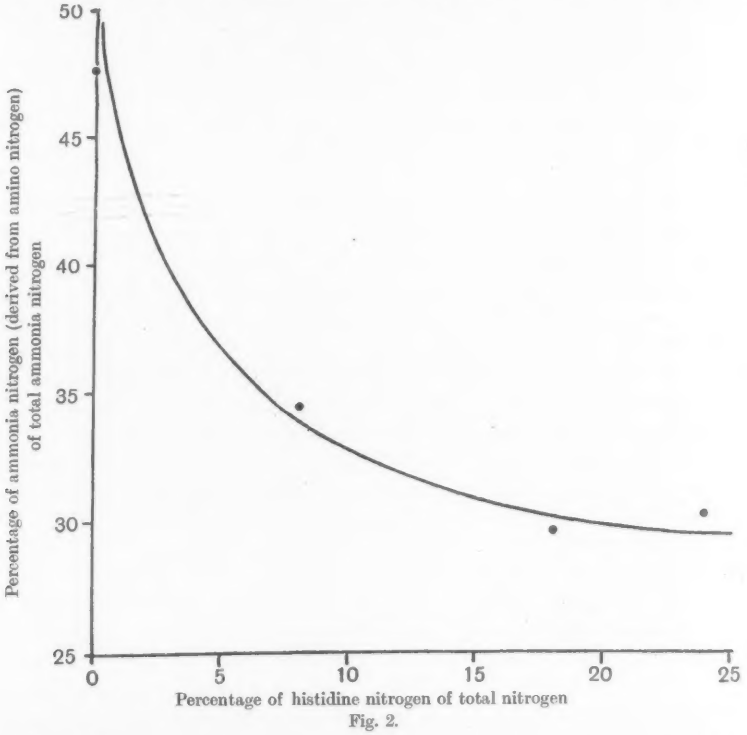

Fig. 2.

2. *Tryptophan and purine bases present in the trypsin employed.*

It was thought essential to make an estimation of these substances. For this purpose, 13·25 g. of Merck's trypsin was dissolved in $N/10$ sodium acetate, and the clear solution transferred to a Winchester quart bottle of 2650 cc. capacity. 2 g. of sodium fluoride (previously dissolved and filtered) were added, and the whole made up to about 2600 cc. with $N/10$ sodium acetate. The p_H was adjusted to 8·1 with caustic soda solution, and the enzyme digest placed in an incubator at 38°. After 20 hours, the digest had become acid to cresol-red and the p_H was again adjusted. This procedure was continued until the digest ceased to change, and, after four days, it was then filtered. In order to maintain the concentration of enzyme as near as possible to that employed in the original solution, 2 litres of the filtrate were added to 8 litres of 7 % sulphuric acid, thereby obtaining 10 litres of fluid of about 5 % acid. 1200 cc. of the Hopkins and Cole reagent were added, and the precipitate allowed to stand for four days. The remainder of the filtrate (about 600 cc.) was treated separately but, otherwise, in all particulars like the main bulk.

The latter, smaller, precipitate was also filtered off separately and used for a qualitative test for tryptophan with the glyoxylic reagent. A fairly strong reaction was given. The larger precipitate from the bulk of the filtrate was filtered through hardened filter paper, and then washed with the usual wash-fluid (3 % mercuric sulphate in 7 % sulphuric acid). No tyrosine could be detected in the washings. The filtrate from the larger precipitate was allowed to stand for a further 14 days; a slight precipitate was deposited, but gave no test for tryptophan.

The mercury precipitate from the 2 litres of digest (which was equivalent to 10 g. of trypsin) was washed with water, suspended and treated as usual with hydrogen sulphide, etc. The filtrate was reduced *in vacuo* to 100 cc. The total and amino nitrogen were then determined; the results are given on p. 83.

The purine bases were then estimated as follows: 50 cc. of the above 100 cc. obtained from the decomposition of the mercury precipitate were diluted to 100 cc. The purines were first precipitated with copper by the method of Krüger and Schmid [1901], that is, 6 g. of sodium acetate, 10 cc. of 40 % sodium bisulphite and 15 cc. of 10 % copper sulphate were added. The resulting precipitate was allowed to stand for two days. It was then filtered off, washed free from sulphate, suspended in water made slightly acid with hydrochloric acid, boiled and treated with hydrogen sulphide. The sulphide precipitate was filtered off and washed free from chloride. The combined filtrates were boiled to remove hydrogen sulphide and made up to 100 cc. In this the purines were then precipitated with silver by the method of Arnstein [1897], that is, 10 cc. of magnesia mixture, 10 cc. of 20 % ammonia and 10 cc. of 1 % ammoniacal silver nitrate were added. The resulting precipitate was allowed to stand in the dark for 24 hours. It was then filtered off, washed free from ammonia, and boiled with a little magnesium oxide to remove the last traces of ammonia;

the total nitrogen of the whole precipitate was then determined (Kjeldahl). The results, both for tryptophan and purine content, were as follows:

	Total nitrogen mg.	Total amino nitrogen mg.	Total purine nitrogen mg.	Total nitrogen other than purine mg.	Total non-amino nitrogen mg.
10 g. trypsin	11·231	4·252	8·021	3·210	6·979

From the above it will be seen that the total non-amino nitrogen in the 2 g. of trypsin used is only 1·396 mg. As it is impossible with so small an amount to apportion the nitrogen between purines, tryptophan and other possible substances present, this correction has been abandoned.

3. *Proline precipitated by the Hopkins and Cole reagent.*

This has already been shown [Onslow, 1921] to amount only to 10 mg. in 200 g. of commercial caseinogen, *i.e.* 2·5 mg. in 50 g. From pure caseinogen it is probably less.

It has therefore been decided to omit the corrections for nitrogen introduced by proline and by the trypsin extract. They probably only partially compensate for the inevitable loss of some tryptophan during the estimation.

Sincere thanks are due to Prof. Hopkins, F.R.S., for his kind help and stimulating advice throughout the research.

Some apparatus used in the research was provided by the Medical Research Council. A small proportion of the total expenses was defrayed by a grant from the Royal Society.

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X. DISTURBANCES IN METABOLISM.

I. VARIATIONS IN PROTEIN METABOLISM AS INDICATED BY SULPHUR EXCRETION.

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(*Received October 26th, 1923.*)

According to the generally accepted theory of Folin [1905, 2] protein metabolism is more or less sharply divided into two fractions; the exogenous metabolism, representing the circulating amino-acids which have not yet been built up into tissue-proteins, and the endogenous metabolism representing the breakdown of tissue-proteins. On this theory the former will vary and the latter remain constant with varying intake of protein, unless the intake of protein is reduced to such an extent that a starvation effect is obtained. In support of this theory Folin pointed out that certain of the urinary constituents, *e.g.* creatinine, and the neutral sulphur, remain constant under various dietary conditions, whilst others, *e.g.* urea and inorganic sulphate, vary directly with the amount of nitrogen and sulphur in the diet. He assumed, therefore, that the creatinine and neutral sulphur in the urine indicated the amount of tissue breakdown.

In conditions where the total metabolism, as indicated by the basal metabolic rate, is raised or lowered, it seems of interest to investigate the partition of protein metabolism, with a view to determining whether the increase or decrease in metabolism is due to an alteration in the amount of tissue breakdown, or of exogenous oxidation, or of both.

Assuming the truth of Folin's theory, we undertook the investigation of some cases of disordered metabolism using the partition of sulphur excreted in the urine as an index of the protein metabolism. The cases investigated were principally those of patients suffering from exophthalmic goitre together with a few cases of reduced metabolism, three of fever, and one of starvation. At the outset of the investigation, it was hoped that the absolute amount of neutral sulphur excreted in the day would afford an index of the condition, but this did not turn out to be the case. Not only does the amount of neutral sulphur excreted by different normal individuals show considerable variations, so that there is no standard to which reference can be made [Folin, 1905, 1], but we soon found also that the amount of neutral sulphur excreted bore no obvious

relation to the rate of metabolism. The estimation of the amount of neutral sulphur in one 24-hour specimen of urine gave therefore no useful information, and it was necessary to devise a more systematic means of attacking the problem.

The method adopted was to observe the reaction of the patient to a change in diet. In certain of the cases, the output of urea and creatinine in the urine was also estimated, in order to compare the nitrogen excretion with that of sulphur.

Experimental.

The method employed for the estimation of sulphur was that of Rosenheim and Drummond [1914] as modified by Fiske [1921] with the following alterations:

(1) Such amounts of urine were taken as would afford a convenient amount of benzidine sulphate for titration.

(2) The benzidine sulphate was titrated with $N/40$ sodium hydroxide, using a microburette, to increase the accuracy of the titration.

(3) Instead of the special filtration-tube recommended by Fiske, we found it convenient to employ a small conical porcelain filter-funnel with a perforated bottom, the precipitate and filter-pad being washed into the beaker for titration by means of a jet of hot water.

(4) The addition of acetone to the solution after treatment with benzidine hydrochloride was discontinued, as it was found that there was a danger of precipitation of some of the reagent[1].

All estimations were carried out in the usual way, viz. inorganic sulphates, total sulphates, and total sulphur were separately determined, and ethereal sulphates and neutral sulphur obtained by difference.

Robison [1922] criticises the method for total sulphur on two grounds:

(*a*) the inconvenience of Benedict's oxidising reagent through spattering;

(*b*) errors introduced through sulphur contained in the gas.

We ourselves found no such difficulty in the use of Benedict's reagent. It was found advisable however to use spirit-lamp oxidation in place of coal-gas as recommended by Robison, his results being confirmed by blank experiments of our own. With the method as finally adopted, using a solution made up of pure sodium sulphate and pure cystine, it was found that it could be analysed in duplicate to within 1 % of the theoretical content. At first we did only three or four estimations on each case, but the results were so remarkable, that, in order to exclude the possibility that the variations we observed were due to inaccuracies in the 24-hour specimens, we decided to carry the experiment over a longer period. We finally adopted a method of more rigid control as follows. After a preliminary period of a few days on ordinary ward diet, the patient was transferred to a constant rich protein

[1] A private communication of one of us with Fiske has revealed that the above observation was due to the fact that we employed absolute acetone whereas he used 95 %. The additional concentration which we used appears to account for the precipitation of the benzidine hydrochloride.

diet of known sulphur content, whose composition is shown in Table I; the patient was then put on a diet, equal in calorific value to the rich protein diet, but in which the main sulphur-containing proteins were replaced by arrowroot and cream. This diet continued for a week, after which the rich protein diet was resumed for a further week, daily estimations being carried out over the whole period.

Table I. *Composition of diet for controlled experiments.*

A. *Rich protein diet.*

Food	Intake in g. per diem	Carbohydrate	Protein	Fat	Content in sulphur (g.)
"Laitproto"	100	—	82·86	0·78	0·68
Egg-albumin	50	—	50	—	0·80
Milk	560	28	18·48	22·4	0·018
Bread	175	92·75	15·75	2·8	0·035
Butter	75	—	—	62·5	—
Potatoes	90	18	3	0·7	—
Vegetables	150	4·5	2·25	—	—
	Total Calories	143·25 573	172·34 689·36	88·98 860·8	1·533 —

Total calories = 2063·18.

In addition 4 g. of cystine were given daily, bringing the total intake of sulphur to 2·593 g. in the day.

B. *Low protein diet.*

For "Laitproto" was substituted 50 g. arrowroot = 168·65 calories.
„ egg-albumin „ 6 oz. cream = 334·8 „
The cystine was omitted.
The other items of the diet *A* remained unchanged.
Sulphur content of low protein diet = 0·053 g. per diem.

As seen from Table I, the daily intake of sulphur during rich protein diet was 2·59 g., and on low protein diet was 0·053 g. The laitproto and egg-albumin were analysed by ourselves, by the method of fusion with sodium peroxide. The figures for the sulphur content of the bread and milk, which contain a relatively very small amount, were from standard text-book sources. In all cases, the possibility of interference with the results by the administration of drugs was carefully excluded. To raise the sulphur content of the rich protein diet and make the change more marked from the low protein diet, 4 g. of pure cystine (analysed) were added to the daily intake of food.

Explanation of Tables.

The results of the experiments are shown in tables as follows:

Table II *a* shows the complete results of all analyses in one case of exophthalmic goitre under rigid dietary control as described and the averages for a second case; also the results of a normal worked out in exactly the same manner; the results in this case have been averaged.

Table II *b* shows the results in seven other cases of exophthalmic goitre. In Case 3, the total duration of experiment was 13 days, and in Case 4, 17 days, but the results in the different periods have been averaged.

Cases 5–9 are the earlier ones, in which we had a few observations only.

Table III contains the results of two cases of reduced metabolism. Case 10 was a definite myxoedema. Case 11 was a patient suffering from mitral stenosis, with enlarged thyroid, and facies suggesting acromegaly; B.M.R. slightly reduced.

Table IV shows the results of four cases (12–15) of fever, and one case (16), of starvation. Case 12 is the same case as Case 2 in Table II *a*, the patient having an acute infection at the end of the last period there shown.

Case 16 was a destitute woman, brought in in a state of starvation, who was observed during her recovery.

In order to save space, the figures for excretion of ethereal sulphates are omitted from the tables.

Table II *a*.

Case 1. Female. Aet. 30. Exophthalmic goitre.

Diet	Day	Creatinine g. in 24 hours	Sulphur as g. SO_3 in 24 hours			% of total sulphur as		Remarks
			Inorganic	Neutral	Total	Inorganic	Neutral	
R.P.D. (*a*)	1	0·58	1·14	0·10	1·27	89·8	7·8	B.M.R. +58 %
,,	2	0·54	1·95	0·08	2·13	91·5	3·8	
,,	3	0·54	4·01	0·24	4·30	93·2	5·6	
,,	4	0·54	3·32	0·17	3·55	93·5	4·8	
,,	5	0·65	3·35	0·30	3·78	88·6	7·9	
,,	6	0·54	4·96	0·25	5·45	83·6	4·6	
,,	7	0·54	4·65	0·37	5·13	90·6	7·2	
L.P.D.	1	—	—	—	Lost	—	—	
,,	2	0·44	1·07	0·18	1·32	81·0	13·6	
,,	3	0·54	1·31	0·14	1·53	87·3	9·3	
,,	4	0·44	0·97	0·09	1·17	82·8	7·8	B.M.R. +48 %
,,	5	0·51	0·97	0·11	1·22	79·5	9·0	
,,	6	0·36	0·73	0·06	0·96	76·2	6·2	
,,	7	0·45	1·00	0·13	1·16	86·2	11·2	
R.P.D. (*b*)	1	0·48	1·53	0·12	1·72	88·9	7·0	
,,	2	0·55	4·70	0·46	5·20	90·4	8·8	
,,	3	0·52	5·58	0·45	6·18	90·3	7·3	
,,	4	0·45	5·25	0·40	5·78	90·8	6·9	B.M.R. +15 %
,,	5	0·50	5·70	0·47	6·34	89·9	7·4	
			Averages.					
R.P.D. (*a*)		0·56	4·06	0·27	4·44	91·5	6·1	
L.P.D.		0·46	1·00	0·11	1·21	82·6	9·1	
R.P.D. (*b*)		0 51	5·31	0·45	5·87	90·4	7·7	

Case 2. Female. Aet. 27. Exophthalmic goitre.

			Averages.				
O.D. 4 days' av.	0·95	1·64	0·50	2·22	74·0	22·5	
R.P.D. (*a*) 6 days' av.	0·96	4·74	0·56	5·45	87·0	10·3	
L.P.D. 7 days' av.	0·75	1·55	0·19	1·87	83·0	10·2	
R.P.D. (*b*) 5 days' av.	0·67	4·61	0·54	5·29	87·0	10·2	

Normal Case. Male. Aet. 14. Convalescent from acute rheumatism.

			Averages.				
O.D. 4 days' av.	1·14	2·36	0·49	3·06	77·0	16·0	
R.P.D. 5 days' av.	1·56	5·27	0·58	6·04	87·0	9·5	
L.P.D. 5 days' av.	1·50	2·25	0·54	2·97	76·0	18·2	

Analysis of Results.

On examination of the results from the cases of exophthalmic goitre, the most obvious point is the striking reduction of the neutral sulphur on low protein diet. The inorganic sulphate is also reduced, but parallel with, and not to a greater extent than the neutral sulphur, so that the percentages of the total sulphur, represented in the two forms, tend to remain the same on both diets, or the percentage of total sulphur as neutral sulphur may even be lower on

Table II *b*.

Case	Diet	Period	Creatinine g. in 24 hrs.	Sulphur as g. SO_3 in 24 hrs.			% of total sulphur as		Remarks
				Inorganic	Neutral	Total	Inorganic	Neutral	
III	O.D.	7 days' av.	0·81	1·34	0·68	2·27	59·0	30·0	Female. Aet. 29. B.M.R.
	L.P.D.	3 ,,	0·75	0·92	0·51	1·63	56·5	31·3	+75 %
	O.D.	3 ,,	1·03	1·71	0·86	2·90	59·0	29·6	
IV	O.D.	7 ,,	1·50	2·96	1·03	4·30	69·0	24·0	Male. Aet. 19. Exoph.
	L.P.D.	4 ,,	1·20	2·42	0·98	3·73	65·0	26·3	goitre
	O.D.	6 ,,	1·51	3·35	1·22	4·96	67·5	24·6	
V	O.D.	3 ,,	—	1·72	0·36	2·28	75·4	15·8	Female. Aet. 30. B.M.R.
	L.P.D.	1 day	—	1·29	0·18	1·95	66·1	9·2	+24 %
VI	O.D.	1 ,,	0·84	0·85	0·18	1·02	80·7	17·5	Female. Aet. 30. Last
	L.P.D.	1 ,,	0·33	0·56	0·11	0·76	73·5	13·8	observation was made
	O.D.	1 ,,	0·49	0·95	0·18	1·19	80·6	14·6	after treatment with
	O.D.	1 ,,	0·79	0·88	0·24	2·23	84·4	10·9	iodine for two weeks
VII	O.D.	1 ,,	0·66	1·40	0·67	2·22	63·1	30·2	Female. Aet. 27
	L.P.D.	1 ,,	0·61	1·22	0·29	1·62	75·3	17·9	
	O.D.	3 days' av.	1·00	1·28	0·47	2·05	62·5	23·0	
VIII	O.D.	1 day	0·65	1·05	0·37	1·47	71·4	25·2	Male. Aet. 33. B.M.R.
	L.P.D.	1 ,,	0·90	1·59	0·27	1·89	84·1	14·3	+37 %
	O.D.	1 ,,	0·45	1·29	0·31	1·72	75·0	18·0	
IX	O.D.	4 days' av.	0·70	1·39	0·48	1·94	71·6	24·8	Female. Aet. 31. B.M.R.
	L P.D.	5 ,,	0·58	0·91	0·37	1·36	66·8	27·2	+71 %. Between
	O.D.	6 ,,	0·91	1·81	0·99	3·08	58·7	32·2	L.P.D. and 2nd O.D. Pt. menstruated

the low protein diet than on the rich. In the normal, on the other hand, it will be seen from the case recorded (and from Folin's work on the subject) the inorganic sulphate falls markedly on the change from rich to low protein diet, and the neutral sulphur remains unchanged. Hence the percentage of total sulphur as neutral sulphur rises.

The output of creatinine does not show such marked variation in the exophthalmic cases as does the neutral sulphur.

In the cases embodied in Table III an entirely different picture is presented. The neutral sulphur on low protein diet tends to increase, while the inorganic sulphate decreases very considerably. The creatinine remains fairly constant throughout.

Table III. *Reduced metabolism.*

Case	Diet	Day	Creatinine g. per diem	Sulphur in g. SO_3 per diem			% of total sulphur as		Remarks
				Inorganic	Neutral	Total	Inorganic	Neutral	
X	O.D.	1	0·55	2·86	0·29	3·23	89·0	9·0	Female. Aet. 17. B.M.R.
	,,	2	0·77	3·69	0·48	4·35	84·8	11·0	−40 %. Myxoedema
	L.P.D.	3	0·85	2·04	0·49	2·65	77·0	18·5	
	,,	4	0·84	1·02	0·51	1·75	58·2	29·2	
	,,	5	0·91	0·91	0·66	1·82	50·0	36·3	
	O.D.	6	0·82	0·77	0·47	1·42	54·2	33·1	
	,,	7	0·86	1·73	0·26	2·13	81·2	12·2	
XI	O.D.	7 days' av.	0·99	1·40	0·44	1·98	70·7	22·2	Mitral stenosis; enlarged
	L.P.D.	4 ,,	0·83	0·93	0·65	1·80	51·7	36·2	thyroid. Facies suggesting
	O.D.	6 ,,	0·85	1·27	0·59	2·09	60·7	28·2	acromegaly, B.M.R. reduced

In Table IV we have a case of starvation (No. 16) which reacts as one would expect to improved diet, the output of neutral sulphur decreasing with the return to normal conditions. The four cases of fever show a marked increase in the output of neutral sulphur during the febrile condition.

The inorganic sulphate and creatinine are also increased. In Case 12, note the preliminary fall in neutral sulphur immediately before the rise of temperature, and compare this with the steady rise in neutral sulphur in Case 15.

Discussion.

As has been mentioned already, Folin found that in normal individuals the creatinine and neutral sulphur remained constant under low protein diet, whereas the inorganic sulphate and the urea were very considerably decreased. We have ourselves worked out three cases, two normal, the other a patient with an enlarged thyroid but normal metabolism, and in these cases we obtained results similar to those of Folin.

In addition, as will be seen from the results, in the normal case in Table II *a*, the output of inorganic sulphate during low protein diet was many times greater than the intake. It is therefore obvious that inorganic sulphate in the normal may originate from endogenous sources (this may also be deduced from Folin's own results). We would, however, agree with Folin that, in the normal, the neutral sulphur remains absolutely unchanged, even on severe changes of diet, short of a starvation effect. From this there seems to be no doubt that the neutral sulphur, in the normal person, represents at least a constant part of the metabolism. It also appears that there is a minimum excretion of inorganic sulphate which may represent a constant part of the endogenous metabolism, and may be derived from the same source as the neutral sulphur. It remains to put forward some explanation of the results in pathological cases.

If the theory of Folin with regard to the origin of the neutral sulphur is correct, the reduction in the output of this constituent during low protein diet in the cases of exophthalmic goitre would seem to indicate that under these conditions the amount of tissue breakdown diminished, but the clinical condition of the patient, the relative invariability of the creatinine output and the basal metabolic rate all seem to show no diminution in the rate of the metabolism as a whole. What then can be the significance of the reduction in neutral sulphur? We have here to deal with a condition widely differing from the normal. In exophthalmic goitre we have reason to believe that the oxidative processes of the body are accelerated, this acceleration being under the control of the thyroid gland, and independent of dietary factors. If, therefore, we reduce the intake of protein appreciably, it follows that tissue-proteins will be oxidised to a greater extent to make up the deficiency of exogenous protein. If this involved a quantitative increase in the tissue-breakdown only, we should expect to find an increase in the output of neutral

sulphur; in actual fact we find the reverse. We suggest the following explanation. Under the abnormally high oxidation potential which obtains in the body in this disease, some of the sulphur-containing products of tissue-breakdown which are normally excreted in the unoxidised form may be further oxidised and excreted in the form of inorganic sulphate. With a liberal protein diet, there would not necessarily be the demand for this further oxidation, but, if the exogenous supply is cut off, the body is faced with a demand for more

Table IV. *Starvation and fever.*

Case	Day	Creatinine g. in 24 hrs.	Sulphur in g. SO_3 per diem			% of total sulphur as		Temp.	Remarks
			Inorganic	Neutral	Total	Inorganic	Neutral		
XII	1	0·79	3·21	0·20	3·73	86·1	5·4	Normal	Case II in Table II *a*.
	2	0·63	1·35	0·25	1·69	79·8	14·8	100·0°	Exophthalmic
	3	0·73	2·75	0·63	3·39	81·1	18·6	100·4°	goitre. Observation
	4	0·86	1·93	0·74	2·78	69·4	26·6	100·0°	made during attack
	5	0·91	1·79	0·53	2·57	69·6	20·6	100·0°	of tonsilitis.
	6	0·61	2·14	0·60	2·83	75·6	21·2	101·0°	Ordinary ward diet
	7	1·03	2·51	0·67	3·43	73·2	19·5	99·5°	throughout
	8	0·69	1·84	0·56	2·46	74·9	22·8	N.	
	9	0·82	1·46	0·64	2·33	62·7	27·5	,,	
	10	0·78	1·68	0·41	2·23	75·3	18·4	,,	
	11	0·53	0·85	0·39	1·36	62·5	28·7	,,	
	12	0·66	1·16	0·32	1·62	71·6	19·7	,,	
XIII	9 days' av.	1·48	2·35	0·33	2·89	81·4	11·4	101·8° to 104·4°	Case of lymphadenoma. Ordinary ward diet throughout
	7 days' av.	0·91	1·33	0·23	1·75	76·0	13·1	N.	
XIV	After 7 days' fever	0·84	1·45	0·29	1·74	83·2	15·1	Max. 103·6°	Case of influenza
	After 10 days' norm. temp.	0·57	0·86	0·14	1·03	83·5	13·6	—	
XV	1	1·76	2·11	0·75	3·24	65·1	23·1	N.	Male. Aet. 24. Normal. Acute infection
	2	1·71	2·30	0·77	3·31	68·4	22·6	N.	occurred during ex-
	3	1·77	1·66	1·11	3·31	50·1	33·5	101·0°	periment
XVI	On admission	0·95	0·80	0·29	1·38	58·4	21·4	N.	Case of starvation.
	After 7 days	0·98	0·88	0·26	1·30	67·7	20·0	,,	Kept on ordinary
	After 14 days	1·08	1·12	0·28	1·60	70·4	17·5	,,	ward diet through-
	After 21 days	0·64	0·66	0·12	0·83	79·5	14·5	,,	out

protein material which must come from endogenous sources. We think that our results show that the first response to this demand is the further oxidation above referred to, shown by the reduced output of neutral sulphur in the low protein diet. There is probably a minimum consisting of substances which cannot under any circumstances be oxidised in the body, and below which the neutral sulphur cannot be reduced. When the demand for oxidisable protein is such that this limit is reached, then increased tissue-breakdown must ensue. We believe, then, that the decrease in neutral sulphur output observed represents the first physiological response of the body to a reduction of the exogenous protein below its minimum requirements in this disease; contrast this with the fact that, in the normal, under the same dietary conditions, the output of neutral sulphur remains constant because, as we take it,

the demand for oxidisable protein is not so great under a normal rate of oxidation. The results in fever and in the case of starvation also illustrate this point. In these cases there is no reason to assume the existence of a specifically higher rate of oxidation, but during the periods of starvation and of fever there is increased tissue-breakdown, and we find correspondingly a higher output of neutral sulphur than is normal for the individual concerned. The preliminary fall in neutral sulphur at the onset of fever in the exophthalmic Case 12 gives rise to an interesting hypothesis. It appears as if, at the onset of fever, the rate of oxidation in the body is suddenly raised, and this rise is followed by a fall below the previous level.

In myxoedema, it is more difficult to explain the results obtained. Possibly the rate of oxidation, always slow in this condition, is influenced more profoundly than in the normal person by changes in diet. On removal of the stimulating influence of a full protein diet, the rate of oxidation would be depressed and the portion of sulphur originating from tissue-breakdown and perhaps even part of that from the food which is normally oxidised, will, in this case, tend to be excreted as neutral sulphur.

If this theory in regard to exophthalmic goitre is correct, it is an interesting deduction that a low protein diet is contra-indicated in this condition; as the rate of oxidation is being kept up by the hyper-functioning of the thyroid, the removal of oxidisable protein taken with the food must lead to an increased demand for such substances from the tissues, and hence to an increased tissue-breakdown, although the first effect is to call into play the reserve physiological mechanism suggested above. We have never observed any ill-effects from giving a liberal protein diet, and, in fact, the basal metabolic rate tends to be lower on the rich protein diet.

Summary.

1. Determinations have been made of the sulphur excretion under various dietary conditions in cases of disordered metabolism.

2. From the results obtained it is deduced that the increase in metabolism in exophthalmic goitre is due primarily to the acceleration of the exogenous oxidative processes, the increase in tissue-breakdown being secondary to this effect.

In conclusion we wish to express our thanks for assistance and advice to Prof. J. C. Meakins, in whose laboratory the work was carried out, and to the Carnegie Post-graduate Research Fund, for the grant of a scholarship to one of us (J. M. C.).

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XI. ON THE PROPERTIES OF CERTAIN S. AFRICAN OILS WITH RESPECT TO THEIR CONTENT OF VITAMIN A.

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(*Received October 31st, 1923.*)

Of late years much evidence has accumulated pointing to the value of vitamin A as a dietary factor, especially in connection with healthy growth in young animals.

By the labours principally of English and American workers, the distribution of the vitamin has been ascertained in a considerable number of natural and commercial products, principally, however, those belonging to the northern hemisphere. Comparatively little is known of the vitamin content of the local produce of other countries, and in a visit to S. Africa in the years 1920–1921 the author became interested in this question from the dietary point of view. In England and America, it has been found that milk and butter are active in growth-promoting properties, but that their value varies much with the previous diet of the cow, being greatest in the case of milk from pasture-fed cows and least in the case of cows on the usual winter feeds [McCollum, Simmonds and Pitz, 1916; Drummond, 1918; Drummond, Coward and Watson, 1921].

To what extent milk and butter may be a source of vitamin A in S. Africa is quite unknown. Over a great part of the interior, the dry climate makes it impossible for the cattle to obtain green grass for more than a small proportion of the year. Shortage of green vegetables is also common in the dry weather. It is conceivable that the brilliant sunshine may in part compensate for the shortage of green food, both for cattle and man. It seems probable, however, that a deficiency of vitamin A may become significant in certain cases, such as that of the native mine workers who spend many hours underground, and whose diet is subject to various restrictions [Delf, 1922]. It is almost certainly also of importance in the case of young white children, brought up on artificial foods, or on such milk as can be obtained in the more remote districts. For such reasons it seemed desirable to test the growth-promoting properties of some common S. African oils.

The method of testing has been that employed for several years as a routine in this kind of work by Drummond, Zilva, Coward and others. Used with due precautions, it has repeatedly been found capable of giving approximately quantitative results [Drummond and Coward, 1920; Zilva and Miura, 1921]. The young rats used were at first obtained from a dealer, but, in accordance with general experience, better results were gained later by the use of rats bred under laboratory conditions upon a fairly liberal diet.

Young rats about 45–50 g. in weight were placed upon an artificial diet consisting solely of ingredients in the following proportions:

Purified rice starch	150 g.
,, casein	60 ,,
Mixed mineral salts	15 ,,
Marmite	15 ,,
Hardened vegetable fat	30 ,,
Fresh lemon juice	15 cc.
Water	150–170 cc.

The salt mixture had the following composition:

Sodium chloride	52 g.
Magnesium sulphate	80 ,,
Sodium acid phosphate	104 ,,
Potassium phosphate	162 ,,
Calcium lactate	390 ,,
Ferric citrate	35 ,,

The work of many investigators has repeatedly shown that after 3–4 weeks on such a diet young rats mostly cease to grow. When the body weight is steady, addition of even a fraction of a drop of cod liver oil[1] to the diet daily causes growth to be resumed. If the hardened vegetable oil is replaced wholly or in part by some other active fat, growth is also resumed with more or less rapidity. The activity of the added fat is thus judged with a fair degree of accuracy.

(1) *Vegetable oils.* The three vegetable oils examined—sesame, mafurreira and castor oil—were found to be entirely devoid of growth-promoting capacity. Castor oil cannot be tolerated by young rats, but after saponification, the remaining unsaponifiable fraction was fed successfully to a group of four young rats which had ceased to grow on the standard diet. No improvement was seen and growth was not resumed. Certain other vegetable oils (examined in the whole nut) were found by Drummond and Coward to possess slight growth-promoting powers, viz. Brazil, Barcelona, walnut, pea, almond and butter nuts [Drummond, 1919, 2].

(2) *Seal oil, Nos.* 1 *and* 2. These oils were received early in January, 1922, but owing to difficulty in obtaining suitable experimental animals, the tests were somewhat delayed. The oils had been prepared by boiling at atmospheric pressure for about 10 hours. The subsequent storage, prior to delivery, was about two months.

Each of these oils was fed to animals to the extent of 10 % of their diet. All the animals receiving them were found to resume growth in a normal way (see Fig. 1). These oils therefore have a considerable value with respect to their vitamin content. Further experiments were not made with them,

[1] This is true only of a first-rate grade of cod liver oil; for variations in quality of cod liver oil, with season, and mode of preparation see Drummond and Zilva [1921, 1922]. An average grade appears to give perceptible growth on about 15 mg., *i.e.* half a drop administered daily.

however, as it appears unlikely that they would ever be sufficiently abundant commercially to be of any economic importance.

(3) *Whale oils, Samples* 1, 2, 3. These are crude whale oils graded according to their preparation. Like the seal oil, quality 1 was prepared by boiling with steam at atmospheric pressure for about 10 hours. The other two oils were extracted at a higher temperature. This grade was heated by steam at a pressure of 50 lbs., the time not being specified. According to Mr Bearpark, this is the only significant difference between the samples.

Young non-growing rats given the standard diet in which the fat had been entirely replaced by whale oil No. 1 or 2 grew extremely well (Fig. 2).

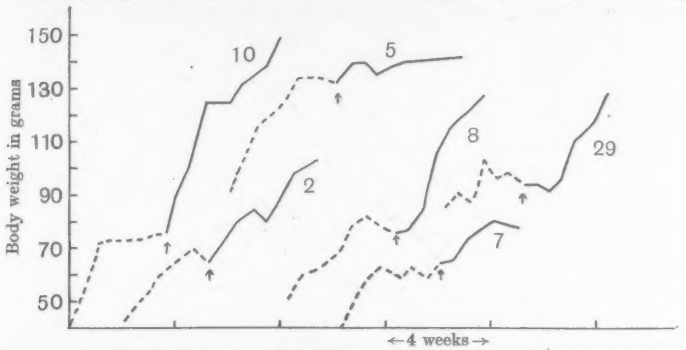

Fig. 1. Chart of growth of young rats at first kept on non-growing diet (broken line). The hardened fat was then replaced by an equal weight of seal oil No. 1, (rats 5, 2 and 10) and seal oil No. 2, (rats 7, 8 and 29) respectively.

Those receiving No. 3, however, were variable in behaviour. Out of nine such animals, four resumed growth at a normal rate; the others after showing a temporary improvement declined in weight, three of these ultimately dying of no apparent disease. This would lead one to suspect a real difference in the vitamin content of the oils, No. 3 being the least valuable in this respect.

When the fat was half replaced by one of the oils, the resulting experiments showed clearly that oil No. 1 was better than oil No. 2 (cp. Fig. 2, Nos. 76, 65 with Nos. 53, 60). With a diet in which oil No. 1 formed only 1 % of the total, three animals out of four showed improvement (cf. Fig. 2, No. 130), one losing in weight (Fig. 2, No. 129). At a later date, four animals were given three drops each of this oil daily as an addition to their non-growing diet, and little or no benefit could be detected, two animals dying and two barely maintaining their weight for the experimental period. This sample appears to have nearly as much activity in growth production as good English butter fat produced in the summer months[1].

[1] It was found by Drummond and Coward that the growth-promoting value of butter fat varies with the season in which it is produced [1920; Coward and Drummond, 1920]. Using a good grade of butter, these workers estimated that 0·2 g. of butter, daily, fed separately to non-growing rats, produces "slow but steady growth" [Coward and Drummond, 1920, p. 735]. With a rat of average appetite on a synthetic non-growing diet, this represents the equivalent of about 1 % of the total synthetic food taken.

If we assume that the whale oils were of similar origin, the extraction at atmospheric pressure (about 100°) appears to be by far the most satisfactory from the point of view of resulting vitamin content, in spite of the greater opportunity for oxidation by this method. Samples of whale oil from other sources previously examined by Drummond were also found to be rich in vitamin A, their relative activity being independent of the "low" or "high" grade [Drummond, 1919, 1].

(4) *Sperm oil (from the head, blubber and meat respectively).* The samples of head and blubber oil were prepared by heating "head matter" and blubber respectively, in an open vessel at 100° for about 10 hours. The meat oil, on the other hand, was heated under pressure at the same temperature as were whale

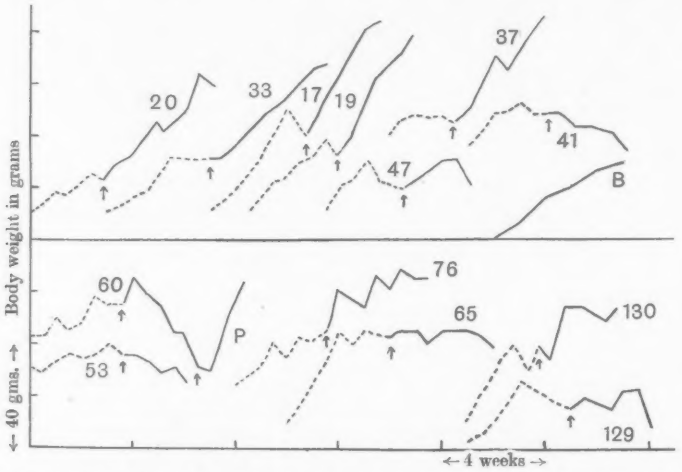

Fig. 2. Chart showing behaviour of typical rats on non-growing diet (broken line) followed by diet containing whale oil.

Nos. 17, 19,	10 % whale oil I	Nos. 53, 60,	5 % whale oil II
„ 20, 33,	„ „ II	„ 65, 76,	„ „ I
„ 37, 41, 47	„ „ III	„ 129, 130, 1 %	„ „ I

No. 60 shows at *P* rapid recovery after transference to a perfect diet (the standard diet in which good butter replaces the hardened fat). Curve *B* represents growth (after a preparatory period on non-growing diet not shown) with a basal diet containing 2 % good English butter; this is constructed from figures kindly supplied by Dr K. H. Coward.

oils 2 and 3. Judging by the results obtained with the three samples of whale oil, much deterioration would be expected by this method.

Each of these oils was first given to experimental animals in the proportion of 10 % of their total diet. When the *head oil* was used as sole source of fat in the diet, a variable but slight improvement was seen (Fig. 3, Nos. 13, 14, 24 and 32). No. 14, however, died soon after the beginning of the experiment.

When either meat or blubber oil was used, however, striking improvement resulted (Fig. 3). The head oil was thus found to be distinctly the least effective of the three oils. Since the method of preparation was not calculated

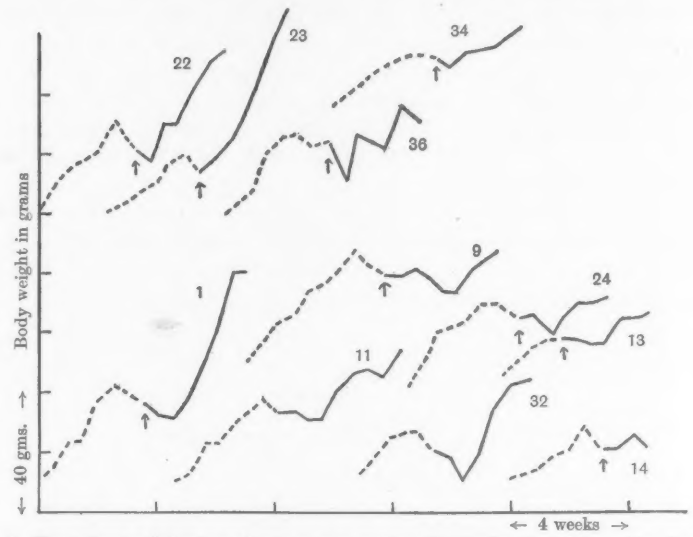

Fig. 3. Chart showing behaviour of rats on non-growing diet (broken line) followed by diet containing 10 % of sperm oil from the head, meat and blubber respectively.

Nos. 22, 23, 34, 36. Blubber oil.
„ 9, 11, 1. Meat oil.
„ 24, 32, 13, 14. Head oil.

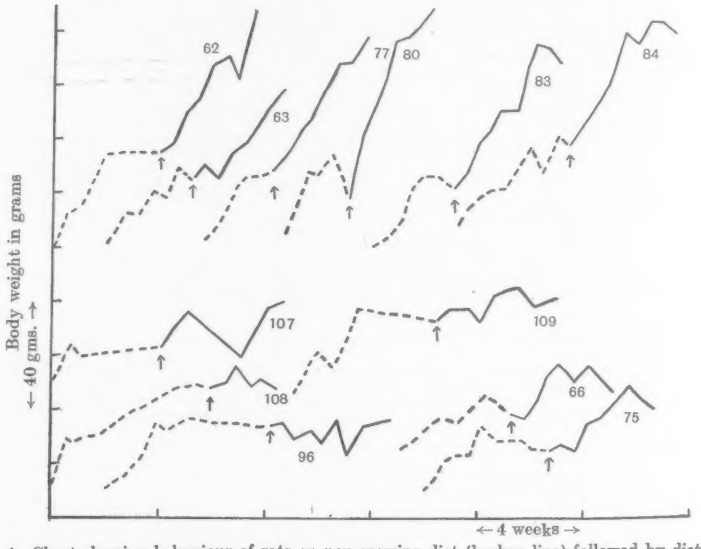

Fig. 4. Chart showing behaviour of rats on non-growing diet (broken line) followed by diet including sperm meat oil.

Nos. 62, 63, 77, 80. 5 % sperm meat oil.
„ 66, 75, 83, 84. 2·5 % „
„ 107, 108, 109, 96. 1 % „

to produce great destruction of the vitamin content, this oil is probably inferior in its original state.

The *sperm meat oil* was also given to groups of four animals in the proportions of 5 % and 2·5 % respectively of the total fat in the diet. In both cases growth was resumed, but those on the larger ration were heavier and more vigorous at the end of the experiment (Fig. 4). On a diet including only 1 % of the oil, three succeeded in maintaining their body weight, and one

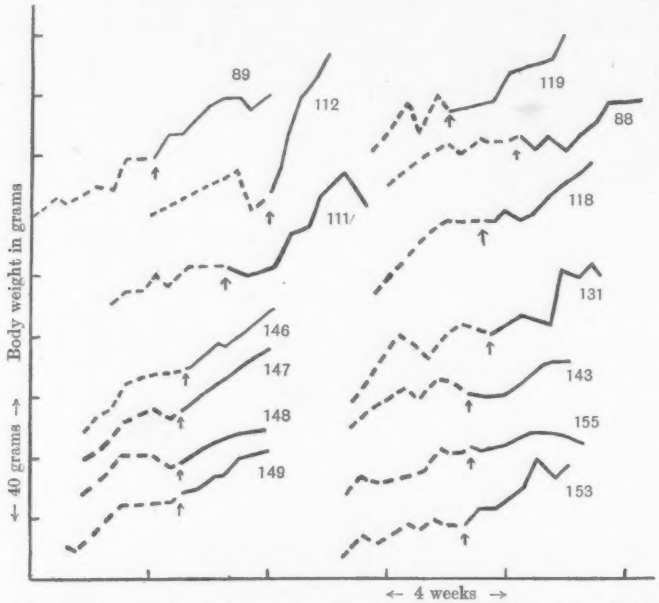

Fig. 5. Chart showing growth of rats on non-growing diet (broken line) followed by diet including blubber oil.

Nos. 89, 111, 112. 1 % blubber oil.
„ 88, 118, 119. 3 drops blubber oil.
„ 146, 147, 148, 149. 3 drops new sample blubber oil.
„ 131, 143, 153, 155. „ „
The basal diet for the last group was made without the addition of fresh lemon juice.

(No. 107) gained somewhat in weight. This oil is therefore very efficient in promoting growth, and appears to have about the same value as good English butter [Cramer, 1922 (cp. Fig. 2, curve *B*)]. Possibly if the method of preparation were modified, the meat oil could be obtained showing still more activity.

Sperm blubber oil was tested in the same way as the meat oil and with diets containing either 5 %, 2·5 % or even 1 % of the oil, good growth resulted. When three small drops were administered daily, in addition to the usual basal diet, a fairly satisfactory growth was obtained (Fig. 5, Nos. 88, 118, 119). When two drops were given daily, growth could still be detected, but when only one drop was given daily, the weight was barely maintained during the

experimental period. By direct determinations, it was found that with the dropping rod used, the drops averaged about ·03 g. each. The drop method ensures that the animals receive their oil ration independently of their appetite for the synthetic food, a point which becomes of more importance as the ration diminishes. With animals of average appetite, a daily dose of three drops is the equivalent of a ration of 1 % of the oil. If the appetite falls off, however, the proportion of oil to the total food taken becomes appreciably greater.

In an ordinary sample of good cod liver oil (cp. footnote, p. 94) about half a small drop daily would give appreciable slow but steady growth [Drummond and Zilva, 1921, 1922].

Sperm blubber oil is thus less efficient than a good sample of cod liver oil in promoting growth, but it is the most active of all the whale oils hitherto examined.

In accordance with the convictions of Harden and Zilva [1918] and of Drummond [1919, 2] that "rats show a more satisfactory growth and development when their ration contains the antiscorbutic factor in addition to the other requisites," the standard diet adopted for the above experiments includes 5 % lemon juice as the source of vitamin C. Certain investigators [Cramer, 1922; Cramer *et al.*, 1922] however still assume that this vitamin is unnecessary for the rat. Cramer states that rats can be bred successfully in the laboratory on a diet of white bread, boiled maize (presumably yellow maize) and rice; he admits that better animals and bigger litters can be produced if cod liver oil and marmite are added to the rations, but does not suggest that fresh green food or carrots would also be beneficial. The antiscorbutic element seems to be in fact excluded from both his normal and experimental diets. This suggests that the need for vitamins A and C may be to some extent interchangeable in the diet of the rat, and in the course of the present experiments a few observations were made which point to the same conclusion.

In Fig. 5 the weight charts of Nos. 146, 147, 148 and 149 represent growth on a diet including three drops daily of blubber oil. The oil was from a second supply received when the first lot had been finished. Nos. 131, 143, 153 and 155 received a similar oil ration, but their basal diet had no lemon juice or other source of antiscorbutic. Those without lemon were more restless and more variable in growth-rate than those with the lemon included. On the whole they made less satisfactory growth. When the same experiment was repeated using a higher ration (2·5 %) of oil, no difference could be detected between the two lots of animals. On the other hand, an increase in the supply of lemon juice does not appear to benefit the rats, whether the supply of oil is minimal or not. When a smaller ration of oil was used (two drops) maintenance of weight resulted without growth, whether lemon juice was supplied or not. Below a certain minimal amount of the fat-soluble factor, therefore, addition of antiscorbutic vitamin (in the form of lemon juice at least) does not appear to compensate for the deficiency of the fat-soluble vitamin.

SUMMARY.

The experiments reported above, have given the following results:

(1) Sesame, mafurreira and castor oils have been shown to be deficient in vitamin A.

(2) Two samples of seal oil are shown to have considerable growth-promoting properties.

(3) A difference in value was found in three different samples of crude whale oil. Sample No. 1 was the most active, and had been extracted (unlike the other two) at 100°. The lower activity of the other two samples was correlated with their extraction at considerably higher temperatures.

(4) The sperm whale gives oils of differing activity with regard to vitamin A. The oil from the head is least and that from the blubber most active in this respect. With more careful extraction, the blubber and meat oil would serve as a very valuable source of this vitamin.

(5) The rat has little need of vitamin C in the diet. It has a much greater need of vitamin A; when the latter is limited however, the addition of vitamin C to the diet appears to have a really beneficial effect on growing rats. This suggests that the two vitamins are to some extent interchangeable in the diet of the rat.

The foregoing experiments were made at the Physiological Institute (Biochemical Department), University College, London, by the courtesy of Prof. J. C. Drummond. My thanks are due to him and to Dr K. H. Coward, for the generous way in which the facilities of the Department were placed at my disposal. The expenses involved were met by a grant from the Board of Scientific and Industrial Research in S. Africa; publication has, however, been delayed for various reasons. Samples of oils were supplied gratis by the respective firms producing them. From the Moçambique Soap and Oils Co. Ltd., of Lorenço Marques, samples of castor, mafurreira and sesame oils were received; and from Irvin and Johnson, Ltd., of Cape Town, various samples of seal, sperm and whale oil. Particulars of these have been supplied by Mr A. H. Bearpark, their scientific oil expert to whom especially my thanks are due.

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XII. NOTE ON THE DETERMINATION BY DISTILLATION OF VOLATILE CONSTITUENTS IN BLOOD, WITH SPECIAL REFERENCE TO THE ESTIMATION OF ALCOHOL.

By HERBERT WILLIAM SOUTHGATE.

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(*Received November 2nd*, 1923.)

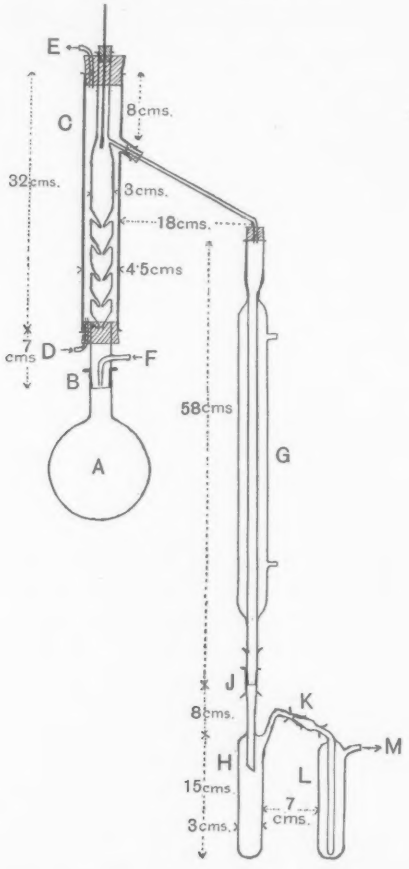
To workers in this field, the frothing of blood on distillation has always proved a difficulty. When, as so often happens, the operation is carried out in an ordinary distilling flask with side tube, it is difficult to obtain a distillate which is absolutely free from haemoglobin, for although no visible drops may be carried over, yet in most cases some of the fine spray generated by the breaking of the blood films passes into the receiver.

The writer during an investigation of the normal volatile reducing substance in blood and also while determining the concentration of alcohol under various conditions in blood, has found the still-head useful which is represented in the accompanying figure. The parts and dimensions of the complete apparatus[1] are given in the hope that they may be of service to others.

The blood or other body fluid is distilled from *A*, a round-bottomed, ring-necked, 1 litre pyrex flask, which is heated in a water-bath (not shown). *A* connects with the lower end of the Lapworth column at *B*, by means of a ground glass joint which is well vaselined to enable the flask to be removed after each distillation. The Lapworth column is surrounded by a glass jacket *C*, in which water at about 75° is placed or made to circulate by the inlet and outlet tubes *D* and *E*. *F* is a tube for introducing (by means of the usual screw clamp and rubber tube) a small current of air to help carry over the vapour into the condenser *G* and also to regulate the pressure. The distillate falls into the receiver *H* which is connected with the lower end of the condenser by a ground glass joint *J*. *H* is connected by means of another ground glass joint *K* with a similar tube *L* which, containing about an inch of distilled water, serves to trap any vapour which has escaped condensation. *M* connects to the water pump to enable the distillation to be carried out under reduced pressure. Tubes *H* and *L* are surrounded by an ice-salt mixture. The upper end of the Lapworth column can be closed by a cork with thermometer if desired. The distillation is carried out with the pressure reduced to 7–8 cm. of mercury. In the case of blood, distillation is carried on till the sticky stage is reached.

[1] Made for me by Mr F. Hartwig of the Scientific Glass Blowing Co., Manchester.

With this still-head there is the great advantage that blood distillations can be left to themselves, for the blood films are rapidly broken up, partly by the heat from the surrounding water in the jacket, and partly by the projections into the lumen of the column. These cones also help to prevent the passing over of fine spray loaded with haemoglobin.



The method and estimation follow in the main those adopted by Pringsheim [1908], who used a modified form of Cotte's [1897] method of estimation. The former heated the solution of alcohol to be estimated with a known amount in excess of $N/20$ potassium dichromate (to which was added 1 cc. of sulphuric acid per 5 cc. of dichromate solution) for 1–1½ hours in the water-bath. After cooling the excess of dichromate was estimated by $N/20$ solution of ferrous ammonium sulphate (to which had been added sulphuric acid to the extent

of 5 % to stabilise). Potassium ferricyanide was used as indicator. In Pringsheim's paper no mention is made of the necessity to have the final volume of the fluid constant before oxidising. It might be expected that this would be an important point, since the extent of the oxidation depends in a large measure on the concentration of the dichromate and sulphuric acid used [see Van Slyke, 1917]. The following figures show the need for this:

cc. of 1 % alcohol taken	cc. standard dichromate	cc. conc. H_2SO_4	Final volume in cc.	cc. stand. ferrous am. sulphate to reduce excess of dichromate
4	20	4	45	8·5
4	20	4	90	10·8
4	20	4	135	14·6
4	20	8	135	9·5

It is evident that as the final volume varies important differences appear in the amounts of ferrous ammonium sulphate used. If the final volume be made the same, both in standardisations and in actual estimations comparable results are obtained as the following tables show:

Table I. *Standardisation of the ferrous ammonium sulphate solution against known amounts of alcohol.*

Equivalent number of cc. of absolute alcohol taken	Equivalent number of cc. of standard ferrous ammonium sulphate solution used
0·01	14·3
0·01	14·2
0·02	28·6
0·02	28·6
0·02	28·6
0·02	28·55
0·03	42·5
0·03	42·4
0·04	56·4

In each of the above estimations 20 cc. of standard dichromate and 10 cc. of concentrated sulphuric acid were taken, the final volume being made up to 150 cc. It is obvious that the estimations are accurate up to quantities of 0·03 cc. of alcohol. In any experiment, if there is reason to suspect a greater amount of alcohol than 0·03 cc. in a distillate the alcohol in a known fraction of this is estimated.

Table II. *The amount of alcohol recovered when known amounts are added to blood and the latter then distilled in the above described apparatus.*

Equivalent number of cc. of absolute alcohol added to blood	Equivalent number of cc. of standard ferrous ammonium sulphate solution required
0·01	14·15
0·02	28·4
0·03	42·4

The alcohol was in each case added to about 10 g. of rabbit's blood.

If blood containing alcohol be allowed to clot it is impossible to recover by the above means the whole of the added alcohol, even if the clot be broken up.

It may be added that the writer finds it much easier to add excess of ferrous ammonium sulphate and titrate this back with standard permanganate solution than to use potassium ferricyanide to determine the end point.

In conclusion it may be stated that it is hoped shortly to publish the results of experiments dealing with the various problems of alcohol metabolism in which the apparatus described is being used.

My thanks are due to Prof. Edward Mellanby for much helpful criticism during the experimental stages of this work. The expenses of this investigation have been defrayed by The True Temperance Research Committee.

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XIII. THE ANALYSIS OF PROTEINS. II. THE ACTION OF NITROUS ACID UPON THE HEXONE BASES.

By ROBERT HENRY ADERS PLIMMER.

From the Chemical Department, St Thomas's Hospital Medical School.

(*Received November 5th, 1923.*)

The estimation of the hexone bases, in the fraction precipitated by phosphotungstic acid, according to Van Slyke's method [1911, 2] depends upon the action of nitrous acid. Lysine loses the whole of its nitrogen, histidine one-third, and arginine one-quarter in the reaction. As the amount of arginine is determined by decomposition with alkali, the amount of histidine can be calculated from the non-amino nitrogen of the solution. The difference from the total nitrogen then expresses the amount of lysine. The values for histidine and lysine are thus obtained indirectly, those for lysine being entirely dependent upon the accuracy of the other two estimations.

The estimation of arginine was investigated by Plimmer [1916] and found to be accurate. Concordant results for the amount of arginine in proteins have since been obtained, but the results for histidine and lysine have never been as concordant as was desired. It thus became necessary to study the action of nitrous acid upon the three hexone bases. Van Slyke [1911, 1] studied the reaction with his original apparatus. He found that the α-amino group of amino acids reacted completely in five minutes. The ϵ-amino group of lysine reacted in half an hour, and in the case of other amino compounds still longer times of reaction were needed for complete evolution of the nitrogen. In the analysis of the hexone bases, Van Slyke stated that a reaction time of half an hour at 20°, or longer at lower temperatures, should be given. No exact time was mentioned for the reaction at the lower temperatures. It was possible, if a longer time was given, that the guanidine complex of arginine might react and cause discrepancies in the estimation.

It has now been found that a time of one hour should be given for the reaction of lysine at temperatures below 20° and that arginine in this time gives amounts of nitrogen in excess of that expected from the α-amino group. This excess is greater in still longer reaction times, which indicates that the guanidine complex reacts slowly. The excess in one hour is very small and not sufficient to cause any appreciable error in the analysis. As far as these three bases are concerned the method is therefore satisfactory.

Experimental.

The large form of apparatus described by Van Slyke [1912] was used. After the air had been expelled from the apparatus, a known volume of solution was introduced into the burette with a pipette and allowed to enter the reaction vessel. The sides of the burette were then washed twice with 1 cc. of water. A control for the determination of air in the reagents was made in a similar way with the same volume of water. In making the determinations it is advisable always to have the same amount of reagent in the reaction vessel; this can be attained by making the reagents stand at a certain mark scratched on the vessel before the solution of amino compound is introduced. The apparatus was shaken continuously for the shorter times of reaction up to one hour; in the case of longer times the reagent and solution were mixed by shaking, allowed to stand and shaken for the last half hour. The evolved gas was treated with permanganate and measured in the usual way. Its volume was then converted to mg. of nitrogen by the table. The control figure was deducted and the amount calculated for the volume of solution made at the start. A total nitrogen determination of the solution was made by Kjeldahl's method, except in the case of lysine picrate, which was weighed out and its amino nitrogen calculated from the formula.

(1) *Action of Nitrous Acid upon Lysine.*

For these experiments a specimen of lysine picrate was kindly supplied by Prof. Drummond. Both the picrate and a solution of lysine prepared from it were used.

(*a*) *Lysine picrate.* 0·4902 g. was dissolved in water containing a small amount of sodium hydroxide and made up to 50 cc. Five or 10 cc. were taken for the estimations. The room temperatures at the time of the experiments varied from 11 to 15°.

Reaction time	N evolved calc. as g. per 10 cc.	Percentage of N	Percentage of N calc. for	
			1 atom N	2 atoms N
15 min.	0·0056	5·71	3·01	7·67
30 „	0·0055	5·61	—	—
3·5 hours	0·0067	6·84	—	—
5·5 „	0·0067	6·84	—	—
15·5 „	0·0076	7·75	—	—
16·5 „	0·0075	7·65	—	—

(*b*) *Lysine solution.* A solution of lysine was prepared from 0·5 g. of the picrate by solution in water, acidification with hydrochloric acid, removal of picric acid by filtration and extraction with ether, separation of ether by evaporation *in vacuo*. The solution was then made up to about 100 cc. Five or 10 cc. were taken for the estimations of amino nitrogen and 10 cc. for that of total nitrogen. The temperature at the time of the experiments was from 15 to 16°.

Reaction time	N evolved in g. per 100 cc.	Total N in g. per 100 cc.
15 min.	0·0278	0·035
30 „	0·0283	—
30 „	0·0304	—
30 „	0·0358	—
1 hour	0·0343	—
1 „	0·0394	—
2·5 hours	0·0364	—
6·5 „	0·0344	—

Thus, in the case of lysine picrate, at temperatures of 11 to 15° complete evolution of nitrogen did not occur until after five and a half hours.

The solution of lysine reacted more rapidly; in one experiment the whole of the nitrogen was given off in 30 minutes. A time of one hour is required for the reaction at 15 to 16° for complete decomposition of the two amino groups.

(2) *Action of Nitrous Acid upon Ornithine.*

In order to confirm the slow reaction of the end amino group of the diamino acids an experiment was made with a solution of ornithine. This was prepared by decomposing arginine carbonate by boiling with 20 % sodium hydroxide for six hours [Plimmer, 1916]. The alkaline solution so obtained was acidified with glacial acetic acid and diluted to 100 cc. Five or 10 cc. portions were used for the amino determinations, which were made at from 14 to 16°.

(1) 0·1540 g. arginine carbonate = 0·077 g. N per 100 cc. ornithine

Reaction time	N evolved calc. in g. per 100 cc.
30 min.	0·0519
45 „	0·0576
60 „	0·0679

(2) 0·07 g. arginine carbonate = 0·035 g. N per 100 cc. ornithine

Reaction time	N evolved calc. in g. per 100 cc.
1 hour	0·0133
2 hours	0·0151
3·5 „	0·0218
17 „	0·031

The whole of the nitrogen was thus not evolved from ornithine in one hour; complete evolution occurred in 17 hours. This long time of reaction is probably connected with the excess of sodium acetate present in the solution derived from the neutralisation of the sodium hydroxide; unfortunately a specimen of ornithine was not available.

(3) *Action of Nitrous Acid upon Histidine.*

A solution of about 0·3 g. of histidine hydrochloride in 105 cc. water was used. Ten cc. portions were taken for the determinations. The temperature varied from 17 to 19°. Total nitrogen was determined in another portion of 10 cc.

Reaction time	N evolved calc. as g. per 100 cc.	Reaction time	N evolved calc. as g. per 100 cc.
5 min.	0·0245	30 min.	0·0267
10 „	0·0251	1 hour	0·0271
20 „	0·0257	1 „	0·0264
30 „	0·0260	17 hours	0·0265
30 „	0·0243		

Total N per 100 cc. = 0·0257 g.

Complete reaction was not attained in 5 minutes at 17°, but in 30 minutes the nitrogen of the amino group was completely given off. There was no reason to expect that reaction would occur with the nitrogen atoms of the glyoxaline ring; this was shown to be the case by the long reaction for 17 hours. The stability of the glyoxaline ring was also confirmed by an experiment with glyoxaline, a specimen of which was kindly supplied by Prof. Barger. No nitrogen was evolved on treatment with nitrous acid under the same conditions.

(4) *Action of Nitrous Acid upon Arginine.*

The same specimen of arginine carbonate as previously used for the study of its decomposition with alkali was employed. Three series of experiments were made:

(*a*) with 0·6 g. dissolved in about 105 cc. water. Temperature from 14 to 17°:

Reaction time	N evolved calc. as g. per 100 cc.	Reaction time	N evolved calc. as g. per 100 cc.
15 min.	0·0375	4·5 hours	0·0501
30 ,,	0·0407	16 ,,	0·0591
30 ,,	0·0390	16·5 ,,	0·0667
1 hour	0·0400	23 ,,	0·0611
1 ,,	0·0406	40 ,,	0·0890
3 hours	0·0497		

Total N per 100 cc. = 0·1540 g. 1/4 = 0·0385 g.

(*b*) with 0·3 g. in about 105 cc. water. Temperature 14 to 17°:

Reaction time	N evolved calc. as g. per 100 cc.	Reaction time	N evolved calc. as g per 100 cc.
·5 hour	0·0267	2 hours	0·0280
1 ,,	0·0294	4 ,,	0·0304
1 ,,	0·0280	21·5 ,,	0·0504

Total N per 100 cc. = 0·1078 g. 1/4 = 0·0267 g.

(*c*) with 0·2 g. in 100 cc. water. Temperature 14 to 16°:

Reaction time	N evolved calc. as g. per 100 cc.
30 min.	0·0173
60 ,,	0·0170

Total N per 100 cc. = 0·07 g. 1/4 = 0·0175 g.

The amino group of arginine thus reacts completely at 14 to 17° in half an hour. In one hour there was a greater evolution of nitrogen and in longer times of reaction the evolution increased progressively. The guanidine nucleus of arginine is evidently slowly attacked by nitrous acid. The excess of nitrogen produced in one hour was small and amounted in one experiment to about 5 %. In carrying out estimations of arginine by this method the time of reaction should not exceed one hour. In this time the other amino acids react completely and any error introduced by excess of nitrogen from arginine is inappreciable.

SUMMARY.

1. Lysine requires a time of one hour for complete reaction with nitrous acid at temperatures from 14 to 17°.

2. The amino nitrogen of histidine and arginine is given off in 15 to 20 minutes at this temperature.

3. A longer time of reaction than one hour has no effect upon histidine, but leads to a greater evolution of nitrogen from arginine.

4. In estimating a mixture of the three hexone bases, it is necessary and sufficient to give a reaction time of one hour at temperatures of 14 to 17° with nitrous acid. The excess of nitrogen given off by arginine will not cause an appreciable error.

Grateful acknowledgement is made to the Government Grant Committee of the Royal Society for a grant to defray the expenses of this work.

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XIV. STUDIES IN THE SULPHUR METABOLISM OF THE DOG. I. THE SYNTHESIS OF ETHEREAL SULPHATE.

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(*Received November 4th, 1923.*)

From the year 1876, when Baumann [1876] first isolated from horse's urine an ethereal sulphate in a pure state and proved it to be potassium phenyl sulphate, the manner in which the synthesis of ethereal sulphate is carried out in the animal body has been a matter of controversy. Baumann thought he had proved in the dog the direct union of phenol introduced subcutaneously with sodium sulphate given by the mouth. The experimental data given in his paper are however too meagre. It is not clear that the ethereal sulphate in the urine arose from the administered sulphate and not from catabolised protein. Sonnenburg [1878] found that sodium sulphate was beneficial in cases of phenol poisoning in man. Schmiedeberg [1881] repeated Baumann's experiments on the dog under slightly different conditions. Instead of administering phenol subcutaneously he gave benzene by the mouth with and without sodium sulphate. The data are again too meagre. The dose of benzene given was very variable and it is quite impossible to make any comparison between the two series of experiments. Marfori [1894] injected dogs with phenol and with phenol together with ammonium sulphate. The output of ethereal sulphate was considerably greater in the second series of experiments.

Tauber [1895] attacked the problem in a different way. He determined the minimum lethal dose of phenol for rabbits and for guinea-pigs and tried to find out if various sulphur-containing compounds afforded any protection against such a dose. The phenol was given subcutaneously and the sulphur compounds intravenously or subcutaneously. He found that sodium sulphate, sodium ethyl sulphate, sodium pyrosulphate, sodium dithionate, sodium bisulphite, sodium thiosulphate and taurine had no beneficial effect. Sodium sulphite had a small beneficial effect. The lethal dose of phenol alone was 0·55 g. per kilo. rabbit, with sodium sulphate 0·66 g. Pfeiffer [1890] had already shown that sulphite was readily oxidised to sulphate in the animal. Boix and Noé [1902] could not find that sodium thiosulphate given subcutaneously protected guinea-pigs in any degree from sodium phenate also injected subcutaneously.

Sato [1909] administered phenol by stomach tube to rabbits and gave also by the stomach various sulphur-containing substances. Isethionic acid did not increase the formation of ethereal sulphate; thio-urea gave a small increase, while cystine and "colloidal sulphur" gave a larger increase. He did not try the effect of sodium sulphate. Granström [1910] could not observe any increase of ethereal sulphate in man after the administration of sodium sulphate in phenol poisoning. Masuda [1910] found that thio-urea caused an increase in neutral sulphur and in ethereal sulphate as well as in inorganic sulphate apart from any phenol administered at the same time. He suggested that phenols are not the only causative factor in the formation of ethereal sulphate. Kenji Kojo [1911] found that thio-urea whether given by the mouth or subcutaneously caused a slight increase in the output of ethereal sulphate, even when phenols were not administered at the same time. He suggested that the thio-urea by oxidation and union with phenols, formed in the gut by bacteria, protected the phenols from further oxidation in the tissues and in consequence the output of ethereal sulphate was increased. Tauber [1895] had previously demonstrated that phenol is capable of oxidation by the tissues.

Rhode's experiments [1922] are more elaborate and greater detail is given. He determined the daily output of inorganic and ethereal sulphate by rabbits, receiving by mouth a daily dose of phenol. On certain days sulphur compounds were administered subcutaneously and the output of inorganic and of ethereal sulphate compared with that on the days on which phenol was given alone. He found that cystine and sodium sulphite increased the output of ethereal sulphate markedly, taurine slightly, sodium sulphate and sodium thiosulphate not at all. Sodium sulphate so administered was readily excreted unchanged without entering into combination with the phenol.

Hopkins [1907] and more recently Sherwin [1922] have suggested that a mercapturic acid, such as is excreted by the dog after a dose of bromobenzene, is an intermediary stage in the formation of ethereal sulphate from phenols and cystine. This last theory will be dealt with in the second and third papers of this series.

As all the sulphur compounds mentioned above, with the probable exception of the mercapturic acids, are oxidised in the animal body to sulphate, some more readily than others, the demonstration of the direct union of any phenol with preformed sulphate would explain any positive effect of these compounds in increasing the output of ethereal sulphate. In the present paper evidence is brought forward of the direct union in the dog of guaiacol and sodium sulphate to form ethereal sulphate.

Experimental.

A fox-terrier bitch, weighing 5 kilos. was used for the experiments. The posterior wall of the vagina was slit open in order to render catheterisation of the urethra easy. She was placed on a standard diet sufficient to maintain body weight. After a period of about 5 days the urine was collected daily

for analysis, the animal being catheterised at least once in every 24 hours. The output of total nitrogen (Kjeldahl), total sulphur, total sulphate and inorganic sulphate (Folin) in the urine was determined daily throughout the experimental periods. Total nitrogen and total sulphur estimations were also carried out on the food and faeces. The standard diet was made up as follows:

		Total nitrogen g.	Total sulphur g.	Calories K
Beef	75 g.	2·56	0·169	100
Suet	10 g.	0·07	0·005	78
Biscuit	60 g.	0·66	0·076	363
Milk	150 cc.	0·78	0·069	107
	Total	4·07	0·319	648

Exp. 1. This experiment lasted for 37 days, from July 1 to August 6, 1919. From July 7 to July 29 4 g. of guaiacol carbonate was administered daily by

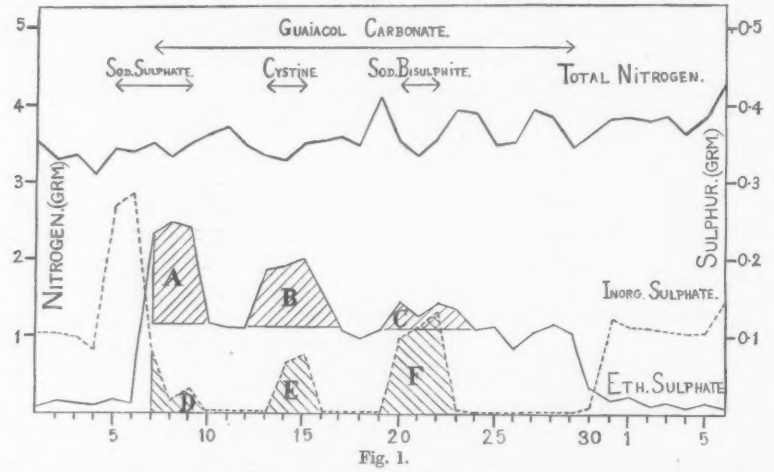

Fig. 1.

the mouth. Guaiacol carbonate, though rather insoluble, was chosen because it is almost tasteless and because it is very little toxic. Probably not more than 1 g. was absorbed daily, the rest passing out unchanged in the faeces. The amount absorbed was very large in proportion to the daily output of sulphur in the urine, so that there was always more than sufficient guaiacol excreted to combine with all the sulphate produced in the course of metabolism. There was a very marked fall in the output of sulphur on the day following the last guaiacol day. This experimental observation will be considered in the second paper of this series.

There was administered by the mouth daily 2 g. sodium sulphate ($Na_2SO_4.10H_2O$) equivalent to 0·199 g. S, from July 5 to July 9; 0·8 g. cystine, equivalent to 0·213 g. S, from July 13 to July 15; and 0·652 g. sodium bisulphite ($NaHSO_3$), equivalent to 0·201 g. S, from July 20 to July 22. The daily output of total nitrogen, inorganic sulphate and ethereal sulphate (total sulphate less inorganic sulphate) is represented graphically above. Neutral

sulphur (total sulphur less total sulphate) has not been shown in the diagram as it was not considered desirable to complicate the figure. The variations in total nitrogen and in neutral sulphur bear little or no relation to the substances given.

EXPERIMENT 1.

Date	Urine							Faeces	
1919 July	Vol. cc.	T.N. g. N	T.S. g. S	In. SO_4 g. S	Eth. SO_4 g. S	N.S. g. S	T.S. × 100 / T.N.	T.N. g. N	T.S. g. S
1	190	3·53	0·158	0·103	0·011	0·044	4·47	—	—
2	190	3·31	0·174	0·102	0·016	0·056	5·26	—	—
3	164	3·34	0·162	0·099	0·014	0·049	4·85	—	—
4	218	3·10	0·156	0·086	0·013	0·057	5·02	1·16	0·144
5	220	3·42	0·339	0·268	0·018	0·053	9·93	—	—
6	176	3·41	0·350	0·281	0·013	0·056	10·27	—	—
7	234	3·50	0·363	0·080	0·232	0·051	10·37	0·61	0·087
8	210	3·32	0·316	0·016	0·249	0·051	9·52	—	—
9	184	3·50	0·320	0·033	0·241	0·046	9·14	—	—
10	188	3·65	0·187	—	0·118	0·069	5·13	—	—
11	210	3·72	0·170	—	0·114	0·056	4·57	1·01	0·146
12	167	3·46	0·156	—	0·114	0·042	4·51	—	—
13	245	3·36	0·249	—	0·185	0·064	7·42	—	—
14	170	3·30	0·327	0·069	0·190	0·068	9·92	—	—
15	276	3·50	0·367	0·077	0·200	0·090	10·49	—	—
16	341	3·53	0·215	—	0·156	0·059	6·10	1·08	0·156
17	113	3·57	0·142	—	0·109	0·033	3·98	—	—
18	156	3·47	0·137	—	0·097	0·040	3·94	—	—
19	214	4·12	0·178	—	0·107	0·071	4·31	0·66	0·067
20	233	3·56	0·308	0·096	0·147	0·065	8·64	0·51	0·073
21	378	3·32	0·305	0·112	0·126	0·067	9·20	—	—
22	257	3·55	0·332	0·135	0·141	0·056	9·35	—	—
23	285	3·90	0·199	0·004	0·136	0·059	5·10	—	—
24	350	3·85	0·178	—	0·110	0·068	4·62	0·73	0·088
25	198	3·48	0·158	—	0·113	0·045	4·53	—	—
26	221	3·52	0·146	—	0·086	0·060	4·14	0·64	0·093
27	252	3·90	0·165	—	0·105	0·060	4·22	—	—
28	184	3·83	0·179	—	0·117	0·062	4·68	—	—
29	208	3·45	0·163	—	0·101	0·062	4·73	1·00	0·093
30	165	3·64	0·091	0·006	0·036	0·049	2·49	—	—
31	279	3·79	0·200	0·122	0·017	0·061	5·28	—	—
August									
1	310	3·79	0·182	0·113	0·019	0·050	4·80	—	—
2	302	3·77	0·174	0·110	0·014	0·050	4·63	1·13	0·162
3	204	3·82	0·166	0·107	0·015	0·044	4·34	—	—
4	224	3·66	0·152	0·101	0·011	0·040	4·16	—	—
5	285	3·79	0·157	0·102	0·014	0·041	4·13	0·80	0·109
6	295	4·04	0·199	0·144	0·010	0·045	4·92	—	—

The dog received daily by mouth:

July	7 to 29, inclusive.	Guaiacol carbonate	4 g.
,,	5 ,, 9 ,,	Sodium sulphate, cryst.	2 g.
,,	13 ,, 15 ,,	Cystine	0·8 g.
,,	20 ,, 22 ,,	Sodium bisulphite	0·652 g.

The weight of the dog was 5·53 kilos. on July 1 and 5·36 kilos. on August 6.

There is a marked rise in inorganic sulphate on the "sodium sulphate" days and an immediate fall on the addition of guaiacol to the diet, with a corresponding rise in ethereal sulphate. The effect of cystine and of bisulphite is less marked on the ethereal sulphate curve, though it is clear that part of the sulphur of these compounds appears in the ethereal sulphate fraction in

combination with the guaiacol. It is noticeable that on the days on which guaiacol is given alone the output of inorganic sulphate falls to zero and that incidentally the output of ethereal sulphate is very constant. In consequence of this fact the area marked *A* represents the administered sulphate in combination with the guaiacol, the area marked *D* the administered sulphate excreted unchanged, the area marked *B* the administered cystine sulphur in combination with the guaiacol, the area marked *E* the administered cystine sulphur excreted as inorganic sulphate, the area marked *C* the bisulphite sulphur in combination with the guaiacol and the area marked *F* the bisulphite sulphur excreted as inorganic sulphate.

If the actual figures from the tables of analyses (p. 113) are taken, the following results are obtained:

	Total ethereal sulphate g. S	Total neutral sulphur g. S	Daily average output of Ethereal sulphate g. S	Daily average output of Neutral sulphur g. S
Control periods:				
First 6 days	0·085	0·315	0·014	0·052
Last 7 days	0·100	0·331	0·014	0·047
Both periods	0·185	0·646	0·014	0·050
Guaiacol days:				
July 10 to 12	0·346	0·167	0·115	0·056
„ 17 „ 19	0·313	0·144	0·104	0·048
„ 24 „ 29	0·632	0·357	0·105	0·059
All 12 days together	1·291	0·668	0·108	0·056

Sodium sulphate. The effect of the administered sodium sulphate is not prolonged beyond the last day on which it is given. From July 7 to July 9 the total output of ethereal sulphate is 0·722 g. S. If the average output of ethereal sulphate for three days on guaiacol alone (0·324 g. S) is deducted, there is left 0·398 g. S, the excess ethereal sulphate due to the effect of the added sodium sulphate. The output of inorganic sulphate for the same three days is 0·129 g. S. The output of neutral sulphur on these days is actually rather less than the normal average.

The results may be summarised as follows:

Sodium sulphate given from July 7 to 9	equivalent to	0·597 g. S
Amount excreted as ethereal sulphate	„	0·398 „
Amount excreted as inorganic sulphate	„	0·129 „
Amount unaccounted for	„	0·070 „

Therefore percentage of administered sulphate excreted in combination with guaiacol is 66.

Similar calculations may be made for the cystine and bisulphite days, though in these instances the effect may be observed over an extra day, *i.e.* the excretion of the sulphate derived from these substances is not so rapid as in the former case.

Cystine days. July 13 to 15.

Cystine given	equivalent to	0·639 g. S
Total ethereal sulphate	„	0·731 „
Less the average output on guaiacol alone	„	0·432 „
Cystine in combination as ethereal sulphate	„	0·299 „
Cystine as inorganic sulphate	„	0·146 „
Cystine unaccounted for	„	0·145 „

Percentage of administered cystine excreted in combination as ethereal sulphate is 47.

The average output of neutral sulphur on the cystine days is 0·070 g. S, a figure rather above the normal average. This may be due to the excretion of the products of bacterial action on the cystine in the intestine.

Bisulphite days. July 20 to 22.

Bisulphite given	equivalent to	0·603 g. S
Total ethereal sulphate	,,	0·550 ,,
Less the average output on guaiacol alone	,,	0·432 ,,
Bisulphite in combination as ethereal sulphate	,,	0·118 ,,
Bisulphite as inorganic sulphate	,,	0·347 ,,
Bisulphite unaccounted for	,,	0·138 ,,

Percentage of administered bisulphite excreted in combination as ethereal sulphate is 19.

The average output of neutral sulphur on the bisulphite days is 0·062 g. S, not much greater than the normal average.

Exp. 2. This experiment lasted from November 6 to December 12, 1919, with an interval of two days due to the animal missing one meal on November 24. The analyses on the faeces and of total sulphur on the urine were omitted. In other respects the experimental conditions were the same as in Exp. 1. Guaiacol carbonate was administered by mouth from November 12 to December 8, sodium sulphate on November 18, 19, 22, 23, 30, and sodium bisulphite on November 14, 15 and December 2. The dose of sodium sulphate given on November 18 and on November 19 was only one-fifth of the dose given on the other days. By calculation from the analyses the following results are obtained:

Fore period, Nov. 6 to 11. Average daily output of ethereal sulphate 0·010 g. S
After period, Dec. 10 to 12. ,, ,, 0·013 ,,
Mean ... ... 0·011 ,,

Guaiacol days, Nov. 12, 13, 16, 17, 20, 21, 26 to 29; Dec. 4 to 8.

Average daily output of ethereal sulphate 0·137 ,,

Date	g. S in compound given	g. S Eth. Sulph. excreted	Less av. output on guaiacol days	g. S Eth. Sulph. due to S compound	g. S Inorg. Sulph. excreted from S compound	g. S in compound given, unaccounted for	Percent. used of S in compound
Sodium bisulphite:							
Nov. 14, 15	0·402	0·349	0·274	0·075	0·304	0·023	18
Dec. 2, 3	0·201	0·388	0·274	0·114	0·058	0·029	56
Sodium sulphate:							
Nov. 18, 19	0·080	0·352	0·274	0·078	0·025	—	about 69
Nov. 22, 23	0·398	0·273	0·274	Nil	0·436	—	Nil
Nov. 30, Dec. 1	0·199	0·382	0·274	0·108	0·092	—	54

On November 18, 19 and on November 22, 23, the amount of sulphur apparently derived from the dose of sodium sulphate was in excess of the dose given. Such discrepancies must occur, when the figures are based on a deduction of an average value. The experiment on November 22, 23 with sodium sulphate was entirely negative. There was no evidence that sodium metabisulphite was less or more effective than sodium sulphate. For the variations in results some other cause must be sought.

Exp. 3. This experiment lasted from October 9 to 27, 1921. The urine was collected in 48 hour periods. In other respects the conditions were the same as in Exp. 1. Guaiacol carbonate 4 g. daily was administered by mouth from October 13 to 27. Sodium sulphate 2 g. daily was given by mouth from October 17 to 23.

EXPERIMENT 2.

Date 1919 Nov.	Urine Vol. cc.	T.N. g. N	In. SO_4 g. S	Eth. SO_4 g. S	Date 1919 Nov.	Urine Vol. cc.	T.N. g. N	In. SO_4 g. S	Eth. SO_4 g. S
6	198	3·64	0·133	0·009	25	—	—	—	—
7	200	3·26	0·126	0·011	26	221	4·30	—	0·130
8	300	3·15	0·111	0·016	27	202	3·75	—	0·141
9	157	3·22	0·117	0·013	28	222	3·76	—	0·135
10	200	3·38	0·119	0·006	29	233	3·91	—	0·145
11	315	3·67	0·137	0·005	30	277	3·88	0·092	0·205
12	227	3·67	0·022	0·129	Dec.				
13	220	3·63	—	0·156	1	232	3·57	—	0 177
14	199	3·39	0·141	0·172	2	222	3·54	0·058	0·235
15	200	3·46	0·153	0·177	3	253	3·81	—	0·153
16	222	3·42	0·010	0·140	4	221	3·53	—	0·134
17	228	3·20	—	0 149	5	270	3·06	—	0·112
18	224	3·54	—	0·179	6	218	3·02	—	0·130
19	222	3·82	0·014	0·173	7	273	3·56	—	0·118
20	222	3·67	0·011	0·142	8	231	3·66	—	0·137
21	232	3·81	—	0·155	9	196	3·66	0·031	0·050
22	233	3·97	0·208	0·148	10	217	3·65	0·121	0·015
23	253	4·06	0·228	0·125	11	261	3·51	0·119	0·011
24	—	—	—	—	12	210	3·62	0·117	0·013

The dog received daily by mouth:

Nov. 12 to Dec. 8 inclusive.	Guaiacol carbonate	4 g.	
„ 14 „ Nov. 15 „	Sodium bisulphite	0·652 g.	
„ 18 „ „ 19 „	Sodium sulphate, cryst.	0·4 g.	
„ 22 „ „ 23 „	„ „	2 g.	
„ 30	„ „	2 g.	
Dec. 2	Sodium bisulphite	0·652 g.	

The weight of the dog was 4·99 kilos. on Nov. 6 and 5·10 kilos. on Dec. 12.

EXPERIMENT 3.

Date 1921 October	Urine Vol. cc.	T.N. g. N	T.S. g. S	In. SO_4 g. S	Eth. SO_4 g. S	N.S. g. S	$\frac{T.S. \times 100}{T.N.}$	Faeces T.N. g. N	Faeces T.S. g. S
9–11	334	7·24	0·339	0·215	0·015	0·109	4·68	—	—
11–13	363	7·66	0·373	0·242	0·020	0·111	4·87	0·63	0·132
13–15	400	7·83	0·396	—	0·300	0·096	5·06	0·83	0·155
15–17	389	7·67	0·368	—	0·272	0·096	4·80	—	—
17–19	332	7·68	0·676	0·185	0·379	0·112	8·80	1·07	0·155
19–21	370	7·37	0·700	0·274	0·330	0·096	9·50	—	—
21–23	372	7·27	0·692	0·201	0·391	0·100	9·52	—	—
23–25	333	7·94	0·366	—	0·272	0·094	4·61	0·93	0·145
25–27	264	7·50	0·324	—	0·226	0·098	4·32	0·82	0·139
27–29	300	7·57	0·275	0·082	0·096	0·097	3·63	—	—
29–31	278	7·50	0·289	0·153	0·029	0·107	3·85	0·92	0·129

The dog received daily by mouth:

Oct. 13 to 27, inclusive.	Guaiacol carbonate	4 g.
„ 17 „ 23 „	Sodium sulphate, cryst.	2 g.

The weight of the dog was 6·12 kilos. on Oct. 9 and 6·04 kilos. on Oct 31.

The following results are obtained from the analyses:

The mean output of ethereal sulphate on the first 4 days	0·009 g. S
The mean output of ethereal sulphate on 8 guaiacol days	0·134 „
The total dose of sodium sulphate given over 6 days	1·194 „
The total output of ethereal sulphate on these days	1·000 „
The ethereal sulphate calculated from the output on the guaiacol days to be deducted	0·804 „
The inorganic sulphate excreted over sulphate period	0·660 „
Sulphate unaccounted for	0·338 „
The percentage of administered sulphate used	16

The results of all the experiments may be summarised thus:

Compound given by mouth	Experiment	Total dose g. S	Percentage used
Sodium sulphate	1	0·597	66
	2	0·080	about 69
	2	0·398	Nil
	2	0·199	54
	3	1·194	16
Cystine	1	0·639	47
Sodium bisulphite	1	0·603	19
	2	0·402	18
	2	0·201	56

Discussion.

It would appear from the above experiments that guaiacol and therefore presumably other phenols will unite in the animal body with preformed sulphate, provided the right conditions are present. Even in the above series there is one complete failure to bring about the synthesis. What are the necessary conditions? It must be remembered that phenols are also excreted in combination with glycuronic acid. Tollens [1910] found that in man the greater portion of the carbolic acid excreted is in combination with glycuronic acid, a lesser portion in combination with sulphuric acid, while the greater portion of indole is excreted in combination with sulphuric acid and a lesser portion in combination with glycuronic acid. Further, Knapp [1911] found that in man after the administration of guaiacol carbonate rather more guaiacol is excreted combined with sulphuric acid than with glycuronic acid. Such a difference in behaviour between phenols might account for the failure of Rhode and of others to demonstrate a synthesis between carbolic acid and preformed sulphate.

In any case it is clear that any one phenol is dealt with in many ways, some part is excreted uncombined, some part is oxidised completely, some part is conjugated with glycuronic acid and some part with sulphuric acid. The mixture, which reaches the blood stream and is excreted by the kidney, will depend upon the relative velocities of the several reactions. It is also certain that the permeability of the kidney to the various reacting substances and end-products is very different. To upset the balance it is necessary to increase the effective concentration of one of the reacting substances at the seat of synthesis. In the case under consideration it is the effective concentration of sulphuric acid. Further, it is necessary to consider the organ or organs, in which the syntheses take place. One is inclined to believe that all tissues contain in some degree the enzymes that catalyse the reactions in question. There is, however, strong evidence that the liver is the chief seat of the

syntheses of ethereal sulphate and of glycuronic acid. Baumann [1876] obtained phenol from liver and from blood by distillation, after hydrolysis with hydrochloric acid, of alcoholic extracts, which before hydrolysis yielded no phenol on distillation. The liver contained 19 times more phenol-yielding substance than blood. Christiani and Baumann [1878] found that ethereal sulphates were not produced in the kidney to any appreciable extent. Kochs [1879, 1880] found that liver mush and muscle mush would synthesise phenyl sulphate from phenol and sodium sulphate. Embden and Glaessner [1902] perfused organs with phenol. They obtained considerable amounts of ethereal sulphate from the liver, small amounts from the kidney and lung. The experiments with muscle and intestine were negative. Satta [1908] extracted organs for ethereal sulphate after the administration of phenol. He obtained the highest yield from the liver, lesser amounts from the kidney, stomach, and blood and practically none from muscle. Pelkan and Whipple [1922] have found that extensive liver injury in dogs lessens phenol conjugation and extreme injury to the liver will reduce phenol conjugation to zero. They conclude that phenol conjugation is a function of liver parenchyma cells and of no other body cells.

It is now possible to discuss the reason why some have failed to demonstrate any direct union between phenols and sulphate. If the liver is the chief source of ethereal sulphate, sodium sulphate injected subcutaneously or intravenously is likely to be excreted by the kidney without affecting in any marked degree the concentration of sulphate in the liver cells, whereas substances like cystine and sulphite, which are not readily excreted by the kidney, may reach the liver and be oxidised actually at the seat of the ethereal sulphate synthesis. Further, phenol itself is so toxic that relatively only small doses can be used with impunity and in consequence any change in the rate of conjugation would be very difficult of detection. There will also always be sufficient sulphate present, derived from protein, to meet the immediate requirements of the tissues. Rhode's observations may be explained on these lines. One could hardly expect such experiments as those of Tauber, Boix and Noé, to yield positive results. Phenol in such doses injected subcutaneously would have a fatal result, long before any synthesis could take place. In the experiments described above the large doses of the relatively non-toxic phenol, guaiacol, given orally and the simultaneous oral administration of sodium sulphate constitute conditions under which the direct synthesis of ethereal sulphate is most likely to be demonstrated.

SUMMARY.

1. Sodium sulphate, sodium bisulphite and cystine are partly excreted as ethereal sulphate by the dog, when administered by the mouth together with guaiacol carbonate.

2. Under these conditions sodium sulphate is utilised as readily as cystine or as sodium bisulphite.

My thanks are due to Prof. F. G. Hopkins for his interest displayed in this research and to my wife for valuable assistance in carrying out the experiments.

The expenses of this research were largely defrayed out of a grant from the Royal Society.

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XV. VITAMIN B CONTENT OF WHITE BREAD.

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(*Received July 25th, 1923.*)

It is frequently assumed that white bread is lacking in vitamin B, yet yeast, which is recognised as being a rich source of this vitamin, is used in bread-making and the temperature reached in baking is not such as would destroy the vitamin.

In 1911 Hill and Flack [1911] fed rats on bread and water, and flour and water in order to compare the relative merits of white, standard and wholemeal breads. None of their animals did well and many died. The rats fed on standard bread did better than those on white bread. These observers state that on wholemeal flour and water the rats "can grow, keep in a very fair condition and beget their own kind." A very obvious criticism of this work in the light of modern knowledge is that the diets used were deficient in fat, vitamin A and mineral salts. Hawk, Smith and Bergeim [1921] found that white bread supplemented with butter and inorganic salts was a diet on which rats could live and put on weight. Their experiments lasted 12 weeks. They used two kinds of bread, one of which was made to approximate closely to home-made bread, and the other contained a large excess of yeast (six times as much). Such bread cannot compare with ordinary bread obtained in this country, because it was mixed with half milk and half water and contained "Crisco," thus making the bread into a more complete food.

The rats fed on the extra yeast bread showed a much better growth and were far superior in general physical appearance. Males were used exclusively in this work and therefore there are no comments as to the possibility of reproduction on this diet.

Edie and Simpson [1911], working on pigeons, found that standard bread is far superior to white bread, both for adult and young animals. Many of their birds fed on white bread died and showed peripheral neuritis. The condition, however, could be cured by adding yeast to the diet.

Chick and Hume [1917] state that white flour is deficient in the anti-neuritic vitamin, and if used as an exclusive diet for pigeons will produce polyneuritis. It is, however, possible that some animals are more susceptible than others to lack of vitamin B.

As the result of these experiments it was natural to assume that baker's ordinary white bread lacks vitamin B. Critically examined the work of Hill and Flack cannot be accepted as a criterion, since their diets were deficient in other respects, and the experiments of the American workers throw no light on the subject because they use whole milk in the making of the bread, and this food has itself been shown to contain vitamin B, while those of Chick and Hume concern the vitamin B content of flour, not that of baker's bread which has a considerable admixture of yeast, and so may be expected to contain vitamin B. Consequently it was thought necessary to make experiments on young growing rats giving white bread as the sole source of vitamin B.

Methods.

(*a*) *Animals.* Rats alone were used in the experiments to be described in this paper. The animals were all bred in the laboratory and their mothers had good mixed diets, both during pregnancy and lactation.

It is usual in most laboratories to take rats of 40 g. weight as the maximal size when the experiments start. With the ordinary stock bought from an animal dealer this corresponds to an age of between 30 and 40 days [Donaldson, 1906]. But with the stock used, bred along lines described by Hartwell, Mottram and Mottram [1923], the mean weight of 40 g. corresponds to an age of 21 days, *i.e.* the day upon which we are accustomed to wean them, whereas animals 38–39 days old weigh no less than 70 g. Consequently two sets of experiments have been made,

(1) with animals of the average *weight*; and

(2) with animals of the average *age*, used in other laboratories.

When the special diet was not started until the animals weighed approximately 70 g., the rats were fed, as are the stock animals, on a diet of kitchen scraps and a small amount of bread and milk.

(*b*) *Foods.* The diet consisted of bread, butter and salt mixture with sufficient water to enable the constituents to be well mixed. The proportions were kept constant, *i.e.* 30 g. bread, 3 g. butter, 0·7 g. salt mixture [Hartwell, 1922, 1], 60 cc. water, and the animals were fed so that small amounts were left—*i.e.* they had as much as they would eat. The bread was weighed one day old. Two kinds of bread were used:

1. White bread bought from a baker.
2. Home-made bread.

This bread was made for me by Miss M. D. Hamilton-King, in the Household Work Dept. of this college, and two varieties were available, viz. baking powder bread and yeast bread. The composition of both is given below. White flour (Household) was used in the preparation of both:

Baking powder bread	Yeast bread
3½ lbs. flour	3½ lbs. flour
8 g. salt	8 g. salt
15 g. sodium bicarbonate	20 g. sugar
25 g. cream of tartar	30 g. yeast

(*c*) *Technique.* For the majority of the experiments 12 animals were selected (six of each sex) and kept in a cage together. They were weighed separately each day, except Saturday and Sunday. They had moist food, but no water to drink.

EXPERIMENTS.

I. *Baker's bread.*

(*a*) *Preliminary experiments.*

A. Two male rats fed on bread, butter and salt mixture and water for *one year*. Weight at beginning of experiment 96 g. and 115 g. Weight at end of experiment 325 g. and 374 g.

B. Two male rats fed on bread and butter and water for $8\frac{1}{2}$ months. Initial weights 101 g. and 106 g.; weights increased to 246 g. and 232 g. respectively.

(*b*) *Further experiments.* Diet: bread, butter, salt mixture and water.

A. Animals started at approximately 40 g.

Exp. 1. 8 rats used, 4 ♀ and 4 ♂. Feeding continued $7\frac{1}{2}$ months.

„ 2. 12 rats used, 6 ♀ and 6 ♂. „ 5 „

B. Animals started at approximately 70 g.

11 rats used, 6 ♀ and 5 ♂. Exp. lasted $4\frac{1}{2}$ months.

II. *Baking powder bread—home-made.*

A. Animals started at 40 g.

12 rats used, 6 ♀ and 6 ♂. Exp. lasted 12 weeks.

B. Animals started at 70 g.

12 rats used, 6 ♀ and 6 ♂. Exp. lasted 6 weeks.

III. *Yeast bread—home-made.*

A. Animals started at approximately 40 g.

12 rats used, 6 ♀ and 6 ♂. Exp. lasted 12 weeks.

B. Animals started at approximately 70 g.

10 rats used, 6 ♀ and 4 ♂. Exp. lasted 11 weeks.

Results. The rats were more susceptible to cold than are normal animals. After 2–3 weeks on the diet, the fur came out in patches; practically all the fur came off, but eventually a new coat grew, and this was quite equal to that of the normal rat. Other work now in progress, suggests that this is due to lack in quality (and possibly also quantity) of the protein.

The growth was slow, but fairly regular. The males did less well than the females, and grew more slowly, the reverse of what occurs in normal animals.

The does produced litters; the babies were of low weight and frequently one or more were born dead. Some of the young were reared. (Details of this will be published later.)

The rats fed on baking powder bread did not thrive as well as the others, and in every case the animals put on to the diet at 70 g. had a distinct advantage, *e.g.* their coats did not come out so badly, etc.

IV. *Growth of rats on bread and water.*

As a further test of the vitamin B content of white bread, it was decided to try to keep some animals on bread and water alone. Males were used exclusively because previous experiments have shown that they do less well than the females on a poor diet. It has been shown by various observers that lack of vitamin B is evidenced before any other dietetic disturbances, hence if vitamin B were absent it would be noticed before the effects of any other deficiencies became evident.

The results of the experiment are tabulated below, and offer good evidence for stating that there is an adequate supply of vitamin B in white bread as bought from a baker.

Length of exp. in days	Weight of rat in g.		Gain in g.
	Beginning	End	
27	259	345	86
27	281	315	34
21	302	315	13
21	320	368	48
21	45	59	14
21	47	58	11
21	45	68	23
21	45	55	10
21	44	68	24
21	47	68	21

From these experiments it can be seen that there is sufficient vitamin B in white bread to provide for the needs of the rat. The growth, however, was by no means good and hence it might be suggested that there is a small amount only of vitamin B in such a diet and that if more were added the growth would improve. To test this the following experiment was made.

V. *Growth of rats on white bread, butter, salt mixture, and wheat germ extract.*

The proportions of the bread, butter, and salt mixture were the same as before, but wheat germ extract was used instead of water. The wheat germ[1] was placed in a Winchester quart bottle with warm distilled water (6 g. germ to 100 cc. water) and shaken on a mechanical shaker for $1\frac{1}{2}$ hours. The supernatant liquid was then decanted, and the amount measured. This was boiled to coagulate some of the protein, cooled and the volume made up with distilled water to what it was before boiling. This liquid was filtered and the filtrate used to soak the bread, *e.g.* 60 cc. to 30 g. bread.

This experiment lasted 10 weeks. The condition of the rats was not at all improved by the addition of extra vitamin B. They were just as susceptible to cold and their fur came off in exactly the same way. Several animals were practically bald on their chests and abdomens. The fur grew again, as it did on the other rats fed with bread, butter, salt mixture and water.

[1] I wish to thank Mr C. Thompson of Penhill Close, Cardiff, for providing me with wheat germ.

The growth of the females was practically the same as before, but the males did slightly better. The improvement was *very* slight, and it is possible that it could be explained as being due to the small amount of extra protein added with the wheat germ extract.

VI. *The vitamin B content of wholemeal and white flours.*

It has been shown previously [Hartwell, 1922, 2] that an excess of protein in the lactating rats' diet is detrimental to the sucklings, unless a large proportion of vitamin B is also included. This fact can be utilised to test the vitamin B content of a food, and the following experiments were made in order to see if white and wholemeal flours contained any vitamin B.

Two samples of flour[1] were available. Sample A, wholemeal, was ground on stones, and represents 100 % of the wheat. Sample B, white flour, was made from exactly the same wheat as A, but was to an extraction from clean wheat of about 72·5 %.

Preparation of flour extracts. The flour was mixed to a smooth paste with a small quantity of distilled water, and this paste washed into a Winchester quart bottle. The proportion of flour to water was 12 g. to 100 cc. This mixture was shaken on a mechanical shaker for 1½ hours, and the subsequent preparation was the same as that described on p. 123 for the preparation of wheat germ extract.

Experimental. The mother rats were fed an excess protein diet, one which had been found to produce bad symptoms in the sucklings, and the flour extract was added to this diet. The method adopted was that described in a previous paper [Hartwell, 1922, 1]. Six mother rats were given the excess protein diet, with the addition of extract of A flour, and six more had a similar diet with extract of B flour added.

The babies whose mothers were fed with A extract (from wholemeal flour) did better than those whose mothers had B extract, *i.e.* there is more vitamin B in wholemeal flour than in white flour. This is a very obvious statement, for it is well known that wholemeal flour contains much more germ than white flour, but the point of interest is that two of the six litters whose mothers had B extract survived, whereas such a diet, without the addition of vitamin B, has previously resulted in the death of all litters. These results, therefore, are in agreement with those described earlier in this paper, in which rats grew and showed no symptoms of lack of vitamin B on a diet of baking powder bread, butter and salt mixture.

VII. *Comments.*

i. *Criticism of experimental procedure.*

In most of this work, the rats were kept in metal cages and had access to the sawdust in the tray at the bottom. Steenbock, Sell and Nelson [1923] have shown that rats, kept on a low vitamin B diet are apt to eat their excreta

[1] I have to thank Mr J. McDougall for sending me the flour.

and hence obtain a further supply of the vitamin. This work was not published when the experiments described in this paper were started, otherwise special precautions would have been taken in this respect. However, an extra experiment (not quoted in the text) has been made in which 12 rats (six female and six male) were kept in a cage with wire meshwork floor, and the tray well below the level of the floor of the cage. The sawdust was changed daily, and therefore the rats had no chance of eating excreta. The rats were fed on baker's bread, butter, salt mixture and water, as before, and the growth curves were identical with those quoted in this paper. The experiment lasted 10 weeks.

Also in experiments I, i and ii, and IV, the animals were kept in similar cages.

It is possible that the butter may contain a trace of vitamin B in the butter-milk. In the 10-week experiment referred to above, the animals were fed on "washed butter" (*i.e.* butter stirred with hot water until melted and the fat when cold allowed to drain through muslin and then pressed as free as possible from liquid).

ii. *Comparison with work on similar lines by other workers.*

The experiments of Hawk, Smith and Bergeim are quite parallel with those described in this paper, except that their bread had a higher nutritive value in that a mixture of milk and water was used in the making. In spite of this, a rather better growth curve is given in the author's experiments, which on the whole agree with those of the American workers.

iii. *Explanation of slow rate of growth.*

It is suggested that the slow rate of growth is due to the lack in quality and quantity of protein. Hawk, Smith and Bergeim [1921] obtained far better growth with "extra yeast" bread, which would certainly contain more protein, since yeast contains 11·7 % of protein. The essential amino acids are poorly represented in wheat protein, and experiments now in progress show far better growth when a good protein is added to the diet of bread, butter, salt mixture and water.

iv. *Comparative growth of male and female.*

As a rule the male animal grows more rapidly than the female. It is extraordinary, that, in these experiments the rate of growth of the two sexes is practically equal, if anything the females are the heavier (see composite curves, Fig. 1). When wheat germ extract was added, the males did rather better than the females. Therefore it appears that, on a poor diet, the female can either make a more economical use of what foodstuffs are available, or that she is accustomed to sacrificing herself for the good of her offspring and is therefore less affected than the male by a poor diet.

The individual curves show that the males have not such good growth as the females, especially when the diet is started at an early age.

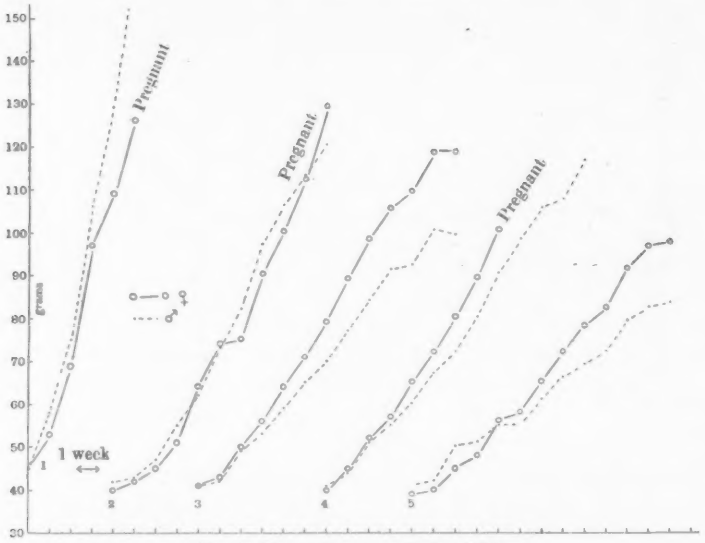

Fig. 1. Composite curves (6 ♂ and 6 ♀).

1. Control animals, fed on bread and milk and kitchen scraps.
2. 30 g. baker's bread, 3 g. butter, 0·7 g. salt mixture, 60 cc. wheat germ extract.
3. 30 g. yeast bread (home-made), 3 g. butter, 0·7 g. salt mixture, 60 cc. water.
4. 30 g. baker's bread, 3 g. butter, 0·7 g. salt mixture, 60 cc. water.
5. 30 g. baking powder bread (home-made), 3 g. butter, 0·7 g. salt mixture, 60 cc. water.

VIII. *Conclusions.*

White bread contains sufficient vitamin B to supply the needs of a rat, both for growth and reproduction. On a diet of white bread, butter, salt mixture and water, the growth is slow, but continuous. This slow growth is probably due to deficiency in the quality and quantity of the protein.

White flour contains a little vitamin B, but the main source is the yeast.

I wish to thank Miss Margaret Dyer for putting her knowledge of types of flour at my disposal and for interesting Mr J. McDougall in the work, Miss M. D. Hamilton-King for making the home-made bread used, and Prof. V. H. Mottram for his unfailing criticism and help.

The expenses of this research were defrayed by a grant from the Medical Research Council.

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XVI. THE CHOLESTEROL CONTENT OF HUMAN MILK.

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(*Report to the Medical Research Council.*)

(*Received November 7th*, 1923.)

The following work on the nature and composition of the "unsaponifiable" constituents of human milk fat was undertaken originally to supply data required for some experiments on cholesterol metabolism in breast-fed infants.

It has long been known that cholesterol, free or in ester form, occurs in human milk, but the quantitative figures published in the earlier literature were little more than estimations of the crude unsaponifiable matter left on hydrolysis of the fat. This so-called unsaponifiable matter consists only in part of cholesterol. The rest consists of low melting oily substances containing oxygen, and of somewhat similar empirical composition to sterols, with perhaps traces of sterol esters. It also contains more or less resinous matter produced in the hydrolytic process [Gardner and Fox, 1921]. It is only in recent years since the development of colorimetric methods and more particularly of the digitonin method of estimating cholesterol, that comparatively reliable quantitative results have been obtained.

In 1918, Denis and Minot published [1918] a series of analyses in which the cholesterol was determined by Bloor's colorimetric procedure [1916], and the fat by a nephelometric method also devised by Bloor. Most of these results refer to late periods of lactation. In Table I we give their figures arranged in descending values of cholesterol content.

In the same year Beumer [1918] published two analyses by the Windaus digitonin method, respectively 0·0105 and 0·011 % total cholesterol.

In 1920 Gamble, using a modification of Bloor's colorimetric method gave, as an average of ten samples, 0·013 %, but without details of individual samples [1920]. The most detailed study has been made by Wacker and Beck [1921] using the digitonin procedure of Windaus. Most of their results refer to late periods of lactation. In Table II we give their results in the order of descending values of cholesterol.

A consideration of the modern literature on the composition of human milk shows that there is some variation in the proportion of the various constituents with the duration of lactation; for instance the percentage of fat appears to increase, of protein to decrease, of the ash to decrease, whilst

Table I. [*Denis and Minot*, 1918.]

Duration of lactation	Fat in 100 cc. milk	Cholesterol in 100 cc. milk	Duration of lactation	Fat in 100 cc. milk	Cholesterol in 100 cc. milk
330	5·4	0·038	14	5·2	0·018
—	6·8	0·031	28	3·6	0·018
330	8·0	0·029	30	4·2	0·018
30	7·2	0·028	330	5·4	0·018
330	8·0	0·028	21	1·8	0·017
90	3·8	0·027	28	4·5	0·016
—	5·2	0·026	60	2·9	0·016
60	6·6	0·026	120	1·2	0·016
60	5·5	0·024	120	2·1	0·016
42	8·0	0·024	49	3·2	0·016
35	7·1	0·024	24	2·4	0·015
63	5·4	0·024	29	1·4	0·015
330	2·5	0·024	25	2·0	0·014
21	—	0·023	28	2·6	0·014
275	5·5	0·023	210	2·2	0·014
30	5·6	0·022	25	1·6	0·014
14	6·8	0·022	5	1·5	0·013
150	2·7	0·021	330	1·5	0·013
240	—	0·020	56	0·8	0·012
14	4·0	0·020	35	0·7	0·011
330	2·7	0·019	—	1·2	0·010
180	2·0	0·019	—	2·3	0·010
Average (44 Exps.):					
113	3·88	0·0190	Cholesterol in 100 g. milk fat 0·490.		

Table II. [*Wacker and Beck*, 1921.]

Duration of lactation	Fat in 100 cc. milk	Cholesterol in 100 cc. milk	Duration of lactation	Fat in 100 cc. milk	Cholesterol in 100 cc. milk
—	3·3	0·036	16	3·4	0·014
14	2·3	0·019	17	3·9	0·013
46	3·4	0·016	315	3·5	0·013
44	3·7	0·016	345	2·8	0·012
210	3·7	0·016	280	3·5	0·012
37	2·6	0·015	345	4·1	0·012
39	2·7	0·015	290	4·6	0·012
14	3·3	0·015	270	3·0	0·011
45	3·5	0·015	315	3·4	0·011
15	3·7	0·015	315	3·1	0·010
290	4·3	0·015	345	1·9	0·007
345	3·7	0·014	345	2·1	0·007
280	3·5	0·014	280	3·0	0·007
Average (26 Exps.):					
200	3·29	0·0139	Cholesterol in 100 g. milk fat 0·424.		

the percentage of sugar remains more or less constant. This however is only apparent when averages of a large number of analyses are considered, since individual variations are large, and the differences of the averages of different lactation periods are within the range of the individual variations on which the averages are based. A difficulty met with in trying to arrive at, for instance, an average fat content by comparing the results of different ob-

servers, arises from the fact that when samples of milk are taken consecutively from the same breast, a more or less marked increase in fat content is observed. This is well known in the case of cow's milk, but does not appear to have been sufficiently taken into account by all writers on human milk. Further the percentages of fat from different individuals under apparently similar conditions vary much more markedly than in the case of animals such as the cow, which long generations of domestic use and systematic milking have no doubt modified.

It seemed to us of interest to find out whether any variation of cholesterol or cholesterol ester content with the duration of lactation could be detected, and how far the cholesterol content varied with progressive milking.

Experimental.

Methods of analysis.

All our analyses were made on the fat as separated by the well-known Gottlieb-Rose method.

The estimations of the non-ester and ester cholesterol and of the total cholesterol were made by the digitonin method fully described in previous papers by one of us, and particularly in our paper on cow's milk [1923].

Sampling.

As many of our results were required for metabolic experiments on infants, samples were drawn before and after nursing and mixed. In other cases the milk was withdrawn in the morning as completely as possible by the breast pump. A great difficulty in dealing with the human subject is that one cannot obtain the milk with the same systematic regularity and under the same controlled conditions as in dealing with an animal. The ideal method would be to work mainly on the milk from wet nurses, but these cannot easily be obtained in this country, so that one is at some disadvantage compared for instance with one's French confrères.

Results.

In Table III we give the results of our experiments in descending order of cholesterol.

Discussion of Results.

It will be seen from the above tables that whereas the bulk of our own estimations were made on milk in the early days of lactation—the so-called colostral milk—those of other observers were mainly on milk at later periods. It seemed therefore that by combining all the results quoted an answer to the questions whether the sterols vary with duration of lactation, and whether there is any relation between fat and cholesterol could be obtained.

The milk during the early days of lactation is known as colostral milk and differs from the later milk both in colour, which is often though *not always*

Table III.

Duration of lactation	Fat in 100 g. milk. g.	Unsaponifiable matter in 100 g. milk. g.	Cholesterol in 100 g. milk. g.	Duration of lactation	Fat in 100 g. milk. g.	Unsaponifiable matter 100 g. milk. g.	Cholesterol in 100 g. milk. g.
30	3·31	0·20	0·047	4	2·18	—	0·027
4	3·80	0·13	0·045	5	2·38	0·10	0·027
8	2·29	—	0·044	—	4·02	0·11	0·027
6	3·07	0·13	0·041	390	2·28	0·06	0·025
6	4·48	0·10	0·039	6	4·02	0·21	0·025
5	2·64	0·32	0·038	253	5·03	—	0·025
10	3·53	—	0·033	3	2·38	0·14	0·024
8	4·43	0·12	0·032	—	2·18	0·13	0·023
254	4·78	—	0·031	249	3·19	—	0·023
—	3·37	0·08	0·029	9	2·05	0·17	0·019
3	1·19	0·12	0·029	7	2·85	0·12	0·019
5	1·24	0·19	0·029	10	1·78	0·14	0·014
6	1·46	0·18	0·027	18	2·59	0·26	0·013
248	6·75	—	0·027				
Average (27 Expts.):							
	3·08	0·15	0·0289	Cholesterol in 100 g. milk fat 0·94.			

more or less yellow, and in composition, being less rich in fat, but richer in mineral matter. It is said, moreover, to have some peculiar physiological properties and is generally considered to be of "educational" importance in developing the digestive activities of the child. This colostral milk gradually gives place to "true milk" which remains of a fairly uniform nature throughout the rest of the period of lactation.

Various writers differ somewhat in their definitions of colostral milk and in the date at which they consider it gives place to ordinary milk.

The most thorough study of these changes in composition with regard to fat, sugar, protein and ash have been made by L. E. Holt and her collaborateurs [1915]. They distinguish four periods each more or less marked by differences in chemical composition, namely (1) colostral 1–12 days, (2) transitional 12–20 days, (3) mature 30–270 days, (4) late 10–20 months. Adopting this perhaps rather arbitrary classification of Holt, we have grouped the average figures of Denis and Minot, Wacker and Beck, and ourselves in Table IV in which our own results have been adjusted from parts per 100 g. to parts per 100 cc. to make them strictly comparable.

Table IV.

Days of lactation	Denis and Minot: Number of experiments on which average is based	Denis and Minot: Average fat per 100 cc. milk in grams	Denis and Minot: Average total cholesterol per 100 cc. milk in grams	Wacker and Beck: Number of experiments on which average is based	Wacker and Beck: Average fat per 100 cc. milk in grams	Wacker and Beck: Average total cholesterol per 100 cc. milk in grams	Average total cholesterol per 100 g. fat
1–12	1	1·5	0·013	1	3·30	0·036	
12–30	15	3·78	0·017	5	3·32	0·015	
30–270	15	3·61	0·019	7	3·23	0·015	
11–20 (months)	8	4·87	0·024	13	3·35	0·0112	
	Fox and Gardner			Average results of all these observers			
1–12	19	2·83	0·030	21	2·79	0·0295	1·057
12–30	2	3·10	0·031	22	3·61	0·0178	0·494
30–270	4	5·09	0·027	26	3·73	0·0192	0·515
11–20 (months)	1	2·37	0·026	22	3·86	0·0165	0·427

It will be seen from this table, particularly columns 11, 12 and 13, that the early milk is richer in total cholesterol than that of the later periods. The total cholesterol of the 1–12 day milk is higher than the average of the other three periods in the ratio 1 : 0·59.

This is also borne out by the following analyses of milk obtained from three French wet nurses, from whom we obtained the whole day's supply.

Table V.

Period of lactation	Fat in g. per 100 cc.	Total cholesterol in g. per 100 cc.
6th day	3·07	0·041
Intermediate, exact date not known	4·02	0·027
13th month	2·28	0·025

On comparing the data in Tables I and II it will be noticed that the values given by Denis and Minot done by a colour method are on the whole slightly higher than those of Wacker and Beck which were done by the Windaus digitonin method. It has been shown that Bloor's method gives somewhat higher results for blood than the digitonin method and even than other colorimetric procedures, and this is probably the case for milk, since, as we show later, the unsaponifiable matter of milk fat contains, besides cholesterol, oils which give greenish colours with acetic anhydride and sulphuric acid and this must to some extent at any rate modify the cholesterol reaction with these reagents. On the other hand it is conceivable that Wacker and Beck's results may be low owing to diet. From the date of their paper it would seem probable that the samples of milk were obtained and analysed during the period covering the end of the war and the first years after the armistice, when the German people are generally admitted to have suffered from dietetic deficiencies. Generally speaking, diet, *if sufficient*, is known to have but little effect, either quantitatively or qualitatively, on the milk at any rate as far as the main constituents are concerned. As to what would be the effect of a prolonged period of more or less deficient diet, particularly if defective in accessory factors, on the fat-cholesterol content of the milk we have no data upon which to base any opinion. On the other hand the average values for cholesterol in cow's milk obtained by Denis and Minot, Wacker and Beck, and ourselves, quoted in our paper on cows' milk [1923], show close agreement.

Relation of fat to total cholesterol.

From a consideration of the figures given in Tables I, II and III we have been unable to detect any relation whatever between the percentage of fat and total cholesterol in individual cases. From column 2 of Table IV however it will be seen that in the early milk (1–12 days) the average fat value is lower than the average of the later periods in the ratio of 1 : 1·35.

The average percentage of total cholesterol in the fat of the three periods of normal human milk is 0·476. This is considerably higher than the average for cow's milk viz. 0·351 given in our former paper [1923]. The higher per-

centage of cholesterol in the ether extract of the early milk as compared with that from milk of later periods is clearly shown in the last column of the table.

The high cholesterol content of the early milk compared with later milk recalls the changes which occur in the cholesterol content of blood during pregnancy. Hermann and Neumann [1911, 1912] were the first to show that during pregnancy the cholesterol content of the serum is increased, and their observations have been abundantly confirmed by Chauffard, Laroche and Grigaut [1911], Klinkert [1913], Schlimpert and Huffmann [1912] and others. During the last few months of pregnancy the increase above normal is often very marked. As an average of 17 cases, between the seventh and ninth months Klinkert finds 0·2633 % against a normal of 0·18. Hermann and Neumann also showed that the increase or retention of cholesterol during pregnancy was followed by an elimination during the puerperium. The chief means of excretion is the milk and they noted that the state of hyper-cholesterolaemia passed off more rapidly in women who nursed their own children than in those who did not.

Though there is no reason to doubt the truth of these general conclusions, much reliance cannot be placed on the absolute figures obtained by these various workers since the recent work of Fex [1920] has conclusively shown that the methods of extraction of fat and cholesterol from blood and serum hitherto used are open to very grave errors. We hope, however, to return to this subject in a later paper, since E. Pritchard [1922, p. 94] has expressed rather definitely the opinion that "the gradual change from colostrum to milk which occurs between the first day of life and the time when true milk is excreted—generally the third or fourth day—gives the stomach and the intestines time and opportunity to settle down to their newly acquired function. It is impossible to conceive of any nutritive fluid being less irritating to the stomach and intestines than colostrum for, as already stated, it is practically maternal lymph, the same fluid that has bathed the foetal tissue during the previous nine months of intra-uterine life."

Ratio of free or non-ester cholesterol to cholesterol in the form of esters.

Wacker and Beck and also Beumer in his 1918 paper stated that the whole of the cholesterol of milk (in general) is present in the form of esters. In a later paper [1922] however, Beumer gives for a sample of human milk the following figures.

Free cholesterol 0·021 %, total 0·038 and ester 0·017, a ratio of free to ester cholesterol of 1 : 0·81.

We have analysed a number of samples for free and ester cholesterol and the results are given in Table V.

The average ratio of free to ester cholesterol from these results is therefore 1 : 0·53. In our paper on cow's milk we gave the ratio 1 : 0·54. As far as these few results give any information, there appears to be no connection between the ester cholesterol and the fat.

Table VI.

	I	II	III	IV	V
Period of lactation	3 days	?	?	6 days	390 days
Fat in g. per 100 g. milk	2·38	3·77	4·02	3·07	2·28
Total cholesterol, g. per 100 g. milk	0·0235	0·0288	0·0265	0·0413	0·0254
Free " "	0·0115	0·0211	0·0207	0·0280	0·0155
Ester " "	0·0120	0·0087	0·0058	0·0133	0·0099
Ratio of free to ester cholesterol	1 : 1·04	1 : 0·43	1 : 0·28	1 : 0·47	1 : 0·63

Variation of cholesterol content during milking.

It is well-known in the case of the cow that the strippings of milk are much richer in fat than the earlier fractions, though the other constituents are less variable. This appears also to be the case in the human subject, and is well illustrated by the figures due to Schlossmann [1900] given in Table VII.

Table VII.

Breast	Vol. of milk withdrawn cc.	Fat %	N %	Sugar %
Right	77	5·90	0·2	6·8
Left	18·5	4·40	0·14	8·04
	37·0	4·40	0·20	7·15
	26·0	8·47	0·20	7·10
	11·2	10·36	0·21	8·00
	15·6	10·58	0·26	7·98

Denis and Talbot [1919] published analyses of samples of milk taken before and after nursing in 56 cases which show the same phenomenon, though on the whole the differences are, as one might expect, less exaggerated.

It was necessary for the purpose of our metabolic experiments to ascertain whether the cholesterol content before and after nursing showed any marked variation as in the case of fat or was more or less constant as in the case of other constituents.

We found that though the percentage of cholesterol of the milk varies markedly in different individuals, and is not always the same from the two breasts of the same woman; on progressive milking the cholesterol content of the different fractions does not show the regular variation noted in the case of fat. This will be clear from Table VIII.

Table VIII.

Age of baby	Breast	Weight of milk withdrawn in g.	Fat per 100 g. milk in g.	Unsaponifiable matter per 100 g. milk	Total cholesterol per 100 g. milk	Total cholesterol per 100 g. of milk fat
11 days	Right	55 (whole)	1·78	0·14	0·014	0·79
	Left	26	1·14	0·09	0·016	1·40
		24	1·86	0·21	0·022	1·18
		29	2·27	0·10	lost	—
1 month	Right	112 (whole)	3·31	0·20	0·047	1·42
	Left	23	2·61	0·15	0·035	1·34
		24	2·19	0·20	0·028	1·28
		27	3·04	0·16	0·039	1·28
		22	3·99	0·20	0·042	1·05

It seemed therefore justifiable to determine the intake of cholesterol of a suckling child on the basis of analysis of samples of milk taken immediately before and after nursing, but there would be danger of very appreciable error in calculating this intake from average values applied to an individual mother.

Qualitative examination of the unsaponifiable matter of human milk.

In Table III we have given the quantities of unsaponifiable matter obtained by hydrolysis of the ether extract per 100 g. of milk. From these figures it will be seen that the sterol precipitable by digitonin, *i.e.* the cholesterol, constitutes only a fraction of the total unsaponifiable matter. The remainder consists of sticky yellow oils, very inactive and not easy to characterise. The figures for this unsaponifiable matter are very variable and too high, being vitiated from a quantitative point of view by the presence of small variable amounts of resinous matter produced during the hydrolysis of the fat by alkalis in alcoholic solution [Gardner and Fox, 1921]. This error is very dependent on conditions of reaction.

Sterols precipitable by digitonin.

The cholesterol digitonides obtained in our analyses were decomposed in the vapour of boiling xylene as recommended by Windaus. The crystalline matter obtained in this process was recrystallised from alcohol and found to be cholesterol from its crystalline form, its melting point and the properties of its benzoate. We had not sufficient material however to subject it to the elaborate fractional crystallisation which we performed on the similar material obtained from cow's milk.

Oil not precipitated by digitonin.

The golden yellow oil obtained after removal of the cholesterol digitonide was soluble in hot alcohol. On cooling traces of a white gelatinous substance separated. This was recrystallised several times from hot alcohol, and was finally obtained in white flakes which on heating softened at 59° and melted at 62–63°. It gave no sterol colour reactions. There was not sufficient for analysis or further examination.

The oil left after separating the last traces of the above substance by freezing in acetone solution was distilled in a high vacuum from a metal-bath as a mobile amber coloured oil leaving a slight tarry residue. This oil was again distilled in a high vacuum, but with the quantity at our disposal (0·4 g.) it was impossible to determine accurately its boiling point. On treatment in chloroform solution with acetic anhydride and sulphuric acid it gave a dark reddish brown colour which soon changed to a dusky green. On combustion it was found to contain 79·34 % of carbon and 11·64 % of hydrogen.

It was evidently a mixture, but as far as we could ascertain from the small quantity in hand it recalled in properties the similar oils we obtained from cow's milk.

We take this opportunity of expressing our thanks to Dr Ribadeau Dumas, Hôpital de la Maternité, Paris, for his kindness in providing samples of milk from French wet nurses, to Dr Remington Hobbs of the Kensington Infirmary, to Sister Nairne of the General Lying-in Hospital, to Dr Pearce of the Royal Free Hospital, and to our own colleagues at St George's Hospital for collecting samples of milk required. We also thank the Government Grant Committee of the Royal Society for help in defraying some of the cost of the work.

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XVII. ON THE TREATMENT OF CELLULOSE FIBRES AND WOOD WITH CARBON DISULPHIDE AND ALKALI.

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(*Received June 6th, 1923.*)

In working with the swelling test [Fleming and Thaysen, 1921] it has frequently been observed that treated cotton fibres from which admixed alkali has been removed by repeated washing with distilled water continue to give off alkali when stored wet. The rate at which this alkali is given off is not very considerable, but it is continued for a prolonged time, suggesting that a steady breakdown of some compound formed during swelling is taking place on wet storage.

Table I. *The fibres were swelled by* 15 % *NaOH* – CS_2, *then washed free from adhering alkali, and placed in bottles filled with distilled water.*

Kept in distilled water. Each horizontal line represents a fresh change of water.

Days	Bottle 1 Change of p_H	Bottle 2 Change of p_H	Days	Bottle 1 Change of p_H	Bottle 2 Change of p_H
1	5·2–8·0	—	2	Water not changed	5·2–6·3
1	5·2–8·4	5·2–8·5	1	for 75 days	5·2–6·4
1	5·2–8·4	5·2–8·4	2	,,	5·2–7·2
1	Water not changed	5·2–8·5	1	,,	5·2–6·0
1	for 75 days	5·2–8·4	1	,,	5·2–6·0
2	,,	5·2–8·6	1	,,	5·2–6·0
2	,,	5·2–8·6	1	,,	5·2–6·0
1	,,	5·2–8·6	24	,,	5·2–7·7
1	,,	5·2–8·4	7	5·2–7·8 [1]	5·2–6·9
1	,,	5·2–8·6	7	5·6–8·2 [1]	5·6–7·4
2	,,	5·2–8·5	7	5·2–7·8	5·2–6·9
1	,,	5·2–8·4	7	5·2–7·7	5·2–6·9
1	,,	5·2–8·3	5	5·2–7·8	5·2–6·4
1	,,	5·2–7·3	7	5·2–7·5	5·2–6·4
4	,,	5·2–6·3	7	5·2–7·4	5·2–6·5
7	,,	5·3–8·0	7	5·2–7·7	5·2–6·6
1	,,	5·3–7·4	7	5·2–7·3	5·2–6·3
1	,,	5·3–7·4	19	5·2–7·3	5·2–6·9
1	,,	5·3–8·0	9	5·2–5·8	5·2–6·1
2	,,	5·2–7·4	6	5·2–5·8	5·2–6·1
3	,,	5·2–8·2	12	5·1–5·6	5·1–6·2
1	,,	5·2–7·2			

[1] 75, 6 days.

Table I shows the rate of this decomposition. Every care was taken in these cases to exclude the possibility of an increase in the hydroxyl ions through the breakdown of the glass containers in which the fibres were kept. This was done by coating the containers with paraffin wax. The fibres were kept in water which was renewed at frequent intervals. The figures given in the table represent the p_H of the water after contact with the fibres during the time stated[1].

As will be seen the fibres continued to give off alkali even after 177 days of wet storage. Table II, which was a control experiment, shows that cotton fibres treated with 15 % NaOH alone did not give off alkali to any appreciable extent.

Table II.

Stored with tap water. Each column represents a fresh change of water.

Sample	1 day	4 days	6 days	12 days
	Changed p_H from	Changed p_H from	Changed p_H from	Changed p_H from
III	7·5–7·6	7·4–7·5	7·3–7·1	7·2–7·1

The importance of this breakdown from the point of view of the protection of cellulose fibres against destruction by microorganisms is now being investigated, and will be reported on in due course. In view of certain observations made by Harrington [1921] however, it was thought of interest to extend the investigation to the examination of samples of wood which had been treated with the swelling liquor and to report immediately on the results obtained in this direction.

Working with the larva of the shipworm (*Teredo norvegica*) Harrington found that it possesses marked positive chemotropic properties towards dilute acids. According to Harrington, such acids are normally given off from wood, and his paper suggests that their production may be responsible for the attraction of the teredo larva and of the consequent destruction of timber by the shipworm. From the figures given by Harrington it would appear that it is the increase in hydrogen ions which is responsible for the attraction rather than the chemical nature of the acid itself. It was thought, therefore, that a method which would ensure a steady evolution of alkali from the wood, at least sufficient to neutralise any acid given off, might be useful in the protection of timber against destruction by the shipworm.

Some of the experiments carried out in this direction are quoted in Tables III and IV. The pieces of wood, one of which was an oak slab, and the other a piece of spruce, were impregnated as efficiently as possible with swelling liquors and then washed free from the adhering alkali. After this treatment the wood was placed in paraffin coated bottles filled with tap water. The proportion of water to wood was about 500 g. to 10 g.

As control, Table IV, pieces of untreated hard and soft wood were kept in tap water in the same proportions as those of the treated samples.

It will be seen that the treatment with swelling liquors has had a considerable effect and that, in the case of the hard wood in particular, the

[1] All p_H determinations were made by the colorimetric method.

Table III.

Wood stored with tap water. Each horizontal line represents a fresh change of water.

Days	Hard wood treated Change of p_H	Soft wood treated Change of p_H	Days	Hard wood treated Change of p_H	Soft wood treated Change of p_H
1	7·2–8·1	7·2–7·9	19	7·5–8·0	7·5–8·0
1	7·2–7·4	7·2–7·4	1	7·5–7·8	7·5–7·8
2	7·2–7·5	7·2–7·4	4	7·6–7·7	7·4–7·7
1	7·2–7·4	7·2–7·3	4	7·4–7·4	7·6–7·4
1	7·2–7·4	7·2–7·3	10	7·4–7·6	7·4–7·7
1	7·2–7·4	7·2–7·3	5	7·5–7·6	7·5–7·5
2	7·3–7·4	7·3–7·4	14	7·6–7·3	7·6–7·6
3	7·3–7·7	7·2–7·4	7	7·6–7·5	7·6–7·3
1	7·4–7·4	7·4–7·4	—	—	7·6–7·1 [1]
2	7·4–7·7	7·4–7·5	—	—	7·6–7·2 [1]
1	7·4–7·5	7·4–7·6	21	7·6–7·4	7·3–7·2 [1]
2	7·4–7·5	7·4–7·6	19	7·2–7·3	7·2–7·3
1	7·7–7·7	7·7–7·6	9	7·6–7·4	7·6–7·2
1	7·7–7·6	7·7–7·5	6	7·3–7·5	7·3–7·2
1	7·7–7·6	7·7–7·5	11	7·2–7·3	7·2–7·3
1	7·5–7·6	7·5–7·5			

[1] 7, 7, 7 days.

Table IV.

Days	Hard wood untreated Change of p_H	Soft wood untreated Change of p_H	Days	Hard wood untreated Change of p_H	Soft wood untreated Change of p_H
1	7·8–7·0	7·8–7·6	7	7·6–7·2	7·6–7·7
1	7·6–7·3	7·6–7·5	7	7·6–7·0	7·6–7·8
3	7·6–7·1	7·6–7·6	7	7·3–7·0	7·3–7·5
5	7·5–6·7	7·5–7·4	19	7·2–7·0	7·2–7·6
7	7·6–6·9	7·6–7·5	9	7·6–7·1	7·6–7·8
7	7·6–6·9	7·6–7·6	6	7·3–7·1	7·3–7·8
7	7·6–7·1	7·6–7·8	11	7·2–7·1	7·2–7·6

liberation of acid has been more than counteracted by the constant breakdown, accompanied by formation of alkali, of the xanthates formed during swelling. The effect on the woods has not been as marked as on the cellulose fibres. This is not surprising in view of the difference in the physical condition of the two materials. There is little doubt that had a more intimate incorporation of the swelling liquor been possible in the case of the wood samples, a more marked effect would have been obtained.

Conclusions.

It has been shown that it is possible to treat wood in such a manner that the normal tendency of this material to exude acids is reversed or at least neutralised. The effect of the treatment appears to last for a very considerable time.

In view of observations made by Harrington it is suggested that this treatment might possibly assist in the protection of timbers against destruction by the shipworm.

The authors desire to acknowledge their indebtedness to the Department of Scientific and Industrial Research for financial aid in respect of this investigation, and to the Admiralty for permission to publish the results of the experiments which were carried out at the Bacteriological Laboratory of the Royal Naval Cordite Factory, Holton Heath.

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XVIII. STUDIES OF THE BACTERIAL DECAY OF TEXTILE FIBRES[1].

I. VARIATIONS IN THE RESISTANCE OF COTTONS OF DIFFERENT ORIGINS TO DESTRUCTION BY MICROORGANISMS.

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(*Received November 9th, 1923.*)

The swelling test, a method for the quantitative determination of the destruction of cotton fibres caused by microorganisms was described in this Journal by one of the authors in collaboration with Miss Nancy Fleming [1921]. In the paper in question attention was drawn to a number of problems arising out of the application of this method to the study of the bacterial decay of cotton. Some of these problems have since then been more carefully investigated and the results obtained are discussed in the following pages.

Before dealing with these results, however, the authors wish to draw attention to a paragraph in the paper referred to which has, it is thought, given rise to a serious misunderstanding [Denham, 1922]. In discussing the respective merits of the swelling test and the alkali solubility test for the determination of the extent of bacterial destruction of cotton it was stated that "while the swelling test (Table V) registers a steady rise in the percentage of physically damaged fibres, the alkali solubility test (Table VI) remains more or less constant until the destruction of the cotton has reached a point when it is affected chemically and may be easily detected even by the inexperienced eye." It is very much to be regretted that the wording of this paragraph left room for the interpretation that the damage observable by the swelling test was of a physical nature as distinct from a chemical damage, detectable only by the alkali solubility test. This interpretation was not intended and a closer examination of the two tables, V and VI of the paper to which reference is made, will show that no such differentiation is possible. Whether measured by the swelling test or by the alkali solubility test, the nature of the damage is obviously the same. And since the damage must be regarded as a chemical change of the cellulose by the time it is observable

[1] Though this paper is to be regarded as a continuation of two previous papers appearing in the *Biochemical Journal* under the title of "Deterioration of Cotton on Wet Storage" the title for various reasons has been altered as above.

by the alkali solubility test, it must have been of this nature in its early stages, that is, when it was only observable by the swelling test. It should not be assumed therefore, as Denham [1922] has done, that fibres in which the cuticle has been mechanically damaged, will behave in the same way as bacterially damaged fibres, when subjected to the swelling test. In such mechanically damaged fibres the cellulose itself generally has not undergone any change and should therefore behave like that of normal cotton fibres on swelling. That it actually does this has been repeatedly confirmed by the authors in the course of their investigations. It has further been suggested [Denham, 1922] that the swelling test would give fallacious results in cases of cotton fibres which had been attacked by microorganisms deposited in the central canal. From what has been said it will be understood, that, if an attack on the cellulose has been made by microorganisms from the central canal, the cellulose must have been changed in the same way as it would have been had the attack proceeded from without. And in such cases the damaged cellulose should respond in the usual way to the swelling treatment. It has not, unfortunately, been possible to examine fibres which had been attacked from within the central canal (such cases appear to be extraordinarily rare), but where microorganisms have been observed both within the central canal, and on the surface of the fibre, the swelling test has never been observed to fail.

In the publication by Fleming and Thaysen already referred to [1921] it was mentioned that the susceptibility of raw cottons to destruction by microorganisms appears to vary with the country of their origin. Thus American cottons were found to show greater resistance to attack than Egyptian, and these again greater than Indian cottons. This observation has now been confirmed on a total of 15 different American, 15 Egyptian, 35 Indian and 8 Indo-American cottons and the curves shown in Fig. 1 represent the approximate rates of destruction of the three mentioned types as measured by the swelling test. It is very noteworthy that the rate of destruction of each type is practically constant, so much so in fact, that on several occasions it has been possible to identify raw cottons by means of their rate of destruction by microorganisms.

It will be understood that both the constancy of the rate of destruction, and the variation in the susceptibility to attack are observations of considerable theoretical and practical importance, pointing as they do either to a difference in the chemical or physical nature of the fibres of the various types of raw cotton, or to the presence of inhibitory substances in the more resistant forms of fibres. Further, they open up possibilities of improving existing strains of cotton as regards their resistance to destruction by bacteria by the introduction of selective breeding in the propagation of seed. The importance of such work will be appreciated when it is mentioned that the U.S. Department of Agriculture estimate that the annual losses suffered by that country, and due to the bacterial destruction of raw cotton amount to about 70 million dollars.

In considering the question of the presence of inhibitory substances it is convenient to take into account the importance of the presence or absence of food substances, since it may well be that what appears to be an inhibitory action is nothing more than a lack of essential foods. The connection between the presence of food substances and the rate of deterioration of cotton fibres was determined in an experiment which is quoted in Table I. In this experiment an American, an Egyptian and an Indian cotton were wetted with water containing 0·1 % each of peptone and potassium hydrogen phosphate.

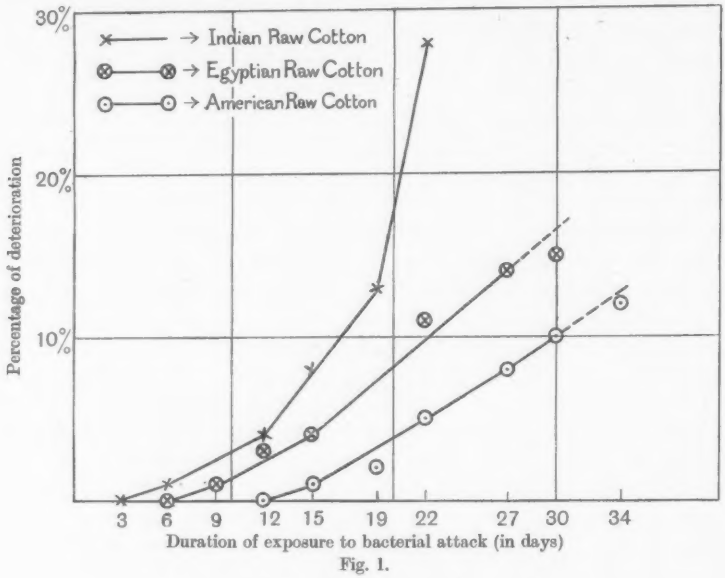

Fig. 1.

Table I.

Sample	Percentage of decomposed fibres found in the cottons after incubation at room temperature, 16°, for 0	6	12	18	24	30	37	42 days
American wetted with food solution	0	1	1	1	2	5	15	21
Egyptian " "	0	1	1	1	4	16	18	35
Indian " "	0	0	3	18	26	31	34	39

American sample = "American Lint." Egyptian sample = "Sakel Combed Sliver."
Indian sample = "Auraiga."

Comparing the figures in Table I with the curves of Fig. 1, it will be seen that the rate of destruction is very little affected by additions of food. Quite a different behaviour, however, is shown by samples of American cotton grown in India. The rate of destruction of two samples of this type was reported on by Fleming and Thaysen in the publication already quoted, and was shown to be practically the same as that of typical American cottons. It has been possible to confirm this on eight further samples of Indo-American cottons,

which were wetted with tap water or distilled water. However, where food substances such as a mixture of peptone and K_2HPO_4 were added, the rate of deterioration of the Indo-American samples increased very considerably and became practically as high as that of ordinary Indian cottons. This is shown in Table II, where the results obtained with a Cawnpore American cotton are tabulated.

Table II.

Sample of Cawnpore American wetted with	Percentage of decomposed fibres in the cotton after incubation at room temperature, 16° for							
	0	6	12	18	24	30	37	42 days
Distilled water	0	0	0	2	1	1	1	2
Tap water	0	0	1	1	3	2	1	4
Food solution[1]	0	—	5	7	8	34	35	44

[1] Containing peptone 0·1 % and K_2HPO_4 0·1 %.

Here, in the case of the Cawnpore cotton, there is no doubt that the addition of food caused an increased deterioration. Similar results have been obtained with other strains of Indo-American cottons, and the resistance of these strains may therefore well be ascribed to the absence from the fibres of sufficient food substances for the development of microorganisms. But it is different with the true American and Egyptian cottons. It has already been shown that the addition of food is without appreciable effect on their rate of destruction, and at one time it was thought that the fibres of these cottons contained substances inhibitory to the growth of bacteria. The experiments which were carried out to prove this were not very conclusive. On the contrary they appeared to indicate that the resistance of the fibres was increased rather than diminished by the treatment to which the samples were subjected for the removal of inhibitory substances. This was the case for instance in the experiment quoted in Table III, in which a sample of Egyptian cotton (Sakel Combed Sliver) was boiled for half-an-hour in distilled water before exposure to the destructive microorganisms, while another sample of the same cotton was treated for three hours with 3 % NaOH at 45 lbs. pressure before infecting. In both of these cases the rate of destruction

Table III.

Sample	Percentage of damaged fibres found in the cotton after exposure to cellulose-destroying microorganisms at 16° for					
	0	6	12	18	25	30 days
A	0	0	6	24	27	33
B	0	0	6	16	21	26
C	0	0	6	13	12	14

A. Sample of Sakel Combed Sliver, damped with distilled water in which food was dissolved (peptone and K_2HPO_4 0·1 % of each), and then infected with cellulose-decomposing micro-organisms.

B. Sample of Sakel Combed Sliver, boiled for 30 minutes in distilled water at atmospheric pressure, then damped with distilled water containing food as above and infected.

C. Sample of Sakel Combed Sliver, heated with 3 % NaOH at 45 lbs. pressure, then mixed with the same food substances as above and inoculated with the cellulose-destroying micro-organisms.

was slower than that of the original raw cotton, though food substances were added to all samples. The above treatment of boiling with water, and with alkali under pressure, was chosen in order to remove inorganic water-soluble compounds (boiling with water) and organic compounds of a fatty or waxy nature (treatment with 3 % NaOH) which might be thought to be capable of preventing the development of microorganisms.

That the higher resistance of the American and Egyptian cottons might be due to the presence of a thicker or more impenetrable cuticle has also been considered. This assumption, however, does not appear justified since (Table III) the treatment with caustic soda at 45 lbs. pressure to which one sample was subjected retarded rather than increased the destruction. Besides, had the presence of a particularly resistant cuticle been responsible for the resistance, it would be expected that, once this obstacle was overcome, the breakdown of the cellulose itself would proceed as fast as in the case of Indian cottons. That this is not the case may be seen in Fig. 1. The greater resistance of the American and Egyptian cottons maintains itself throughout the attack. It appears, therefore, that the cause of the resistance of these cottons is a property of the cellulose itself. Whether it is due to a physical or to a chemical property of the cellulose cannot at present be stated.

One further conclusion may be drawn from the experiments already discussed. The sample of Cawnpore-American cotton which was used for the experiment in Table III and which was found to have lost its resistance in the presence of food substances was a strain propagated by Burt [1921] in pure culture, which had not been allowed to interbreed with pure Indian strains. Its loss of resistance, therefore, must have been caused by climatic or soil conditions.

The value of various food substances for the rate of deterioration of Indo-American cottons has been investigated at some length; the results of the experiments are tabulated in Table IV.

Table IV.

Sample of Cawnpore cotton containing	Percentage of damaged fibres found in the cotton after exposure to cellulose-destroying microorganisms at 16° for 9	12	18	24	30	37	42	48 days
[1]Peptone 0·1 %	0	0	1	4	6	9	11	23
[1]Sodium nitrate 0·1 %	0	0	1	3	5	7	7	10
[1]Pot. hyd. phos. 0·1 %	0	0	1	2	3	3	6	7
[1]Peptone 0·1 %, pot. hyd. phos. 0·1 %	0	5	?	8	34	35	44	—
Control without food	0	0	1	1	1	1	1	5

[1] Calculated on the moisture content of the cotton.

It is of interest to note that nitrogen, and particularly organic nitrogen, increases the rate of destruction of Indo-American cottons very appreciably, though not quite to the same extent as a mixture of phosphates and organic nitrogen. Under the usual storage conditions, Indo-American cottons are, therefore, as liable to deterioration as typical Indian samples, since it is

practically impossible to prevent the contamination of the cotton with traces of organic nitrogen and other food substances such as phosphates.

From the observations discussed it will have been seen that there are two distinct reasons for the resistance of certain raw cottons. It may be due either to the absence of food and particularly of organic nitrogen, or to some unknown property of the fibres. In the first case the resistant cotton might be expected to contain little organic nitrogen or at least considerably less than Indian cottons which are always found to deteriorate rapidly.

Little information is available as to the nitrogen content of raw cottons. Fargher and Withers [1922] quote figures by Schindler; these figures, however, do not include typical Indian cottons. The content of American cottons is given as 0·15 % and that of Egyptian cottons as 0·25–0·257 %. Knecht quoted by the same authors, gives the nitrogen content of an American cotton used by him as 0·208 %.

The results obtained by the authors with Indian and Indo-American cottons are given in Table V.

Table V.

Sample	Origin	Weight of cotton used for determination, g.	Per cent. of nitrogen
Gadag I	Indo-American	18	0·204
,, I	,,	22	0·207
Old Triumph	,,	22	0·167
,,	,,	28	0·157
Cawnpore-American	,,	20	0·111
,,	,,	20	0·123
Indian Cotton	Pure Indian	15	0·215
No. 3 Shippur Factory	,,	27	0·244
M.M.X.	,,	15	0·235

With all the samples the greatest care was taken to remove fragments of leaf and other impurities which had accidentally contaminated the cotton, and the results may be taken to represent the nitrogen content of the clean fibres. Leaving out the Gadag I type (which has been cultivated for a very long time in India under conditions which almost certainly would cause it to interbreed with true Indian varieties and thereby to lose its Indo-American character), there would appear to be a distinctly lower nitrogen content in the Indo-American than in the true Indian types, particularly in the Cawnpore-American sample. The difference, however, does not seem large enough to justify the conclusion that it is a lack of nitrogen which is the sole cause of the resistance of Indo-American cottons. In interpreting the above figures it should perhaps not be forgotten that all the nitrogen found may not be nitrogen available as food. The difference in available nitrogen may possibly be considerably greater than would at first sight appear from the figures in Table V.

It has already been pointed out by the authors [1923] that the swelling test may be used for the study of the breakdown of other fibres besides cotton. Linen fibres for instance have given quite reliable results when subjected to

this treatment. In Table VI figures are tabulated recording the rate of deterioration of two samples of flax, as measured by the swelling test. The two samples had been subjected to a different retting treatment, in the one case termed "dew retted," and in the other "tank retted." Whatever the difference of these treatments, the one would seem to have left the flax much more resistant to bacterial attack than the other.

Table VI.

	Percentage of decomposed fibres found in the flax after incubation at room temperature, 16°, for						
Sample	0	6	12	18	23	30	37 days
English Dew Retted Flax	0	1	2	2	5	7	7
English Tank Retted Flax	0	1	2	7	16	21	21

As regards jute and hemp, these fibres have not been found favourable objects for study by the swelling test. The individual fibres in these cases are too interwoven to make an accurate determination of their rate of deterioration possible.

Conclusions.

Using the swelling test, it is shown that raw cottons of different origin vary in their resistance to destruction by microorganisms. This resistance is probably due to difference in the physical or chemical nature of the respective fibres.

Since the resistance disappears when resistant strains are cultivated in a country producing non-resistant types, it is concluded that the resistance is adversely affected by climatic or soil conditions.

Flax fibres appear to show differences in their behaviour to bacterial destruction similar to those exhibited by cotton.

The rate of deterioration of jute and hemp fibres cannot be determined by the swelling test.

The above experiments were carried out on behalf of the Fabrics Co-ordinating Research Committee of the Department of Scientific and Industrial Research at the Bacteriological Laboratory of the Royal Naval Cordite Factory, Holton Heath. The authors desire to express their thanks to the Department of Scientific and Industrial Research and to the Admiralty for permission to publish the results.

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XIX. IMPROVEMENTS IN THE PREPARATION OF INSULIN. ALKALINE EXTRACTION OF PANCREAS.

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(*Received November 15th, 1923.*)

The original method for preparing insulin from normal fresh pancreas, worked out by Collip [1923], depended on the extraction of the minced tissue with plain alcohol. Doisy, Somogyi and Shaffer [1923] found that they could obtain considerably larger yields of the active principle by extraction with strongly acid alcohol.

The fact that insulin in acid solution is readily adsorbed by a great variety of substances, while in faintly alkaline solutions this is not the case, [Dudley, 1923] suggested that possibly the yield of insulin might be increased by extracting the minced pancreas in an alkaline medium. The only danger which we anticipated was that in such a process we should have conditions favourable to the action of trypsin which is known rapidly to destroy insulin [Dudley, 1923]. Our fears in this respect proved to be groundless and by a very simple modification of the method of alcoholic extraction we have been able to obtain between four and five times the amount of insulin which we had been getting by the original method.

We carry out the process as follows. One kilogram of fresh ox pancreas is minced into one litre of 95 % spirit; 85 g. sodium bicarbonate are stirred into the mixture, which is then poured into the mincer and reminced; this procedure is repeated a second time. The mixture is then allowed to stand with frequent stirring for two hours at room temperature, or if desired it may be kept in the cold room overnight. It is then poured on to a stout cloth, which is squeezed as thoroughly as possible in a suitable press. The turbid filtrate which has a p_H of about 7·5 is treated with $1\frac{1}{2}$ times its volume of 95 % spirit and placed in the cold room (− 3°) overnight. It is then filtered through a folded paper; 10 cc. of glacial acetic acid are added to the filtrate, which is then evaporated *in vacuo*, in a water-bath at 40–45°. When the volume of the residue has reached about 150 cc., the fat which has separated is removed by shaking out with light petroleum and the aqueous layer is freed from traces of this solvent by a few minutes' distillation under the conditions described above.

Four volumes of absolute alcohol are added to the aqueous solution, and after standing for about ten hours in the cold room, the alcoholic solution is carefully decanted from the precipitate which has formed and two volumes of absolute alcohol are added to it. After standing in the cold room for 12–24 hours the supernatant liquid is carefully decanted and the precipitate is washed into centrifuge tubes with absolute alcohol, washed with absolute alcohol and dry ether, and finally dried in a vacuum desiccator over H_2SO_4 as described in a previous paper [Dudley, 1923].

Altogether we have worked up 43 batches of 1 kilo. each by this method. In many of these experiments different amounts of sodium bicarbonate were used and other experimental variations were introduced with a view to finding the best conditions. The yields of crude insulin varied, of course, in these experiments, but, taking the good with the bad, the average yield of insulin over the whole of the series was 257 rabbit units per kilo.

It is fairer to judge the method by taking the results of our last eight batches in which the experimental conditions, described above, were constant. In these the yield of "crude insulin" varied from 1·95 g. to 2·4 g. and the rabbit units from 300 to 480, the average yield of "crude insulin" being 2·16 g. and of rabbit units 412 per kilo. of pancreas.

Our average yield of rabbit units per kilo. pancreas by the original method was 88, that is to say, slightly more than one-fifth of the yield obtained by the alkaline process. The method of acid extraction to which reference has been made above, has, in our hands, given us an average yield of 257 rabbit units per kilo., or about five-eighths of the yield obtainable by the method of alkaline extraction.

A rabbit unit of the crude insulin prepared by alkaline extraction is usually from 3 to 5 mg. In our experience the material obtained by the method of acid extraction is always much more highly pigmented than that prepared by either of the two other processes. An aqueous solution of "acid" crude insulin is a deep brown colour, while a corresponding solution of "alkaline" crude insulin is pale yellow and somewhat less coloured than that prepared by the original simple alcoholic extraction.

The highest yield of crude insulin which we have obtained by alkaline extraction is 508 rabbit units per kilo., and it is worthy of note that the pancreas used was ox-pancreas which was put into the cold room (− 3°) about two hours after removal from the animal, and kept there for a week before it was worked up. It is therefore obvious that, provided it is frozen promptly, pancreas may be stored for a considerable time without risk of losing its insulin.

Improved method of purification of crude insulin.

The crude insulin obtained by alkaline extraction can be purified by precipitation with picric acid and conversion of the picrate into a soluble hydrochloride, as described in the paper previously mentioned [Dudley, 1923].

The technique of this method of purification has been considerably simplified and is now carried out in the following manner. The crude insulin is dissolved in a small volume of water and the flocculent, insoluble material is removed by centrifuging. The clear solution is then diluted with the requisite amount of water to give a 1·5 % concentration of the original material, making no allowance for the insoluble material which has been removed. The p_H of the solution is then adjusted to about 5. This may be conveniently accomplished by adding acid or alkali (according to the original p_H) until a faint turbidity is produced due to the formation of the "isoelectric" point precipitate [Doisy *et al.* 1923]. Half its volume of saturated aqueous picric acid is then added. After standing for a day or two the clear supernatant liquid is poured off. The lemon-yellow precipitate which has collected on the bottom of the beaker is rubbed up with a small amount of water and then $N/10$ sodium carbonate solution is added in small quantities at a time with constant stirring until a clear, or nearly clear, dark brown solution is obtained. As soon as addition of sodium carbonate solution fails to clarify the solution further it is filtered through a folded filter paper, which is well washed with distilled water on completion of the filtration. This process should be carried out with all reasonable speed and it is well to keep the temperature below 10°. The solution is then diluted to contain about 1·5 to 2 g. of picrate per litre. The necessary volume is calculated on the assumption that the amount of picrate obtained is about one-twelfth the weight of the original crude insulin. An amount of $N/10$ HCl, equivalent to the Na_2CO_3 used, is then added to the well-stirred liquid. An immediate precipitation of the picrate occurs. To each litre of solution is added 250 cc. of saturated aqueous picric acid. After standing for a day or two the precipitate has again collected on the bottom of the beaker and the clear supernatant liquor is poured away.

A solution containing 5 cc. of saturated aqueous picric acid in 100 cc. water is made. The picrate is rubbed up in a small quantity of this solution and is then filtered on a Buchner funnel. After adequate washing with the dilute picric acid solution to remove sodium chloride, the moist picrate is transferred to a beaker and stirred with a solution of hydrochloric acid in 75 % alcohol, prepared by mixing 25 cc. 3 N (aqueous) HCl with 75 cc. of absolute alcohol.

The picrate, after first forming thick, dark brown sticky drops, dissolves in this reagent, yielding a turbid, brown solution. It must be stirred until all the sticky drops have disappeared. The amount of the alcoholic-aqueous HCl reagent used is not important but from 10–15 cc. per g. of picrate is an appropriate quantity. Pure acetone is then added rapidly until no further precipitate appears. From 10–20 volumes are necessary. The white flocculent precipitate of "insulin hydrochloride" settles rapidly and is immediately filtered on a Buchner funnel, washed with acetone and finally with dry ether until perfectly free from picric acid. The funnel with its contents is quickly transferred to a vacuum desiccator and dried over sulphuric acid.

The material so obtained is a white, non-deliquescent, amorphous powder, which dissolves completely in water giving a colourless solution.

This method of handling the picrate obviates the necessity of preparing it in the dry state and, further, gives a hydrochloride which contains no trace of picric acid, a result which was difficult to attain by the original method. We have prepared from "alkaline" crude insulin a hydrochloride by this process of which 0·2 mg. proved to be a rabbit unit.

In the course of these experiments we have found that while the picrate is only very slightly soluble in water and practically insoluble in absolute alcohol it is quite freely soluble in mixtures of water and alcohol. It must therefore be emphasised that insulin cannot be precipitated satisfactorily by means of picric acid from aqueous solutions containing alcohol; in the absence of alcohol, however, it is precipitated quantitatively.

Wherever "rabbit units" are mentioned in this paper, we mean the original Toronto rabbit unit; *i.e.* the amount of insulin necessary, when injected subcutaneously, to lower the blood sugar of a 2 kilo. rabbit which has fasted for 16–24 hours, from a normal value of about 0·1 % to about 0·04 % and to cause typical hypoglycaemic convulsions within four hours.

Summary.

1. By a simple modification of the original process of alcoholic extraction of pancreas, namely, by adding sodium bicarbonate to the mixture of minced pancreas and alcohol the yield of insulin is increased nearly fivefold. In one experiment 508 rabbit units were obtained (as finished hydrochloride) from 1 kilo. of ox pancreas.

2. An improved technique for the purification of crude insulin by precipitation with picric acid and subsequent conversion of the picrate into a soluble hydrochloride is described.

3. At least 90 % of the impurities in crude insulin are removed by this method.

4. By these means preparations have been obtained of which 0·2 mg. has proved to be a "rabbit unit."

We wish to express our best thanks to Mr H. P. Marks, who has carried out all the physiological assays of the insulin produced in this series of experiments.

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XX. CHOLINE AS A PRECURSOR OF GUANIDINE. THE DECREASE IN THE AMOUNT OF CHOLINE OF THE HEN'S EGG DURING INCUBATION.

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(*Received November 19th, 1923.*)

Guanidine not only occurs in the protein molecule as arginine but it is also found in muscle in simpler combination as creatine—methylguanidineacetic acid—and also as methylguanidine [Smorodinzew, 1923].

In tetania parathyreopriva Burns and Sharpe [1917] found the amount of methylguanidine markedly increased in the blood and urine and in idiopathic tetany, in the urine.

The source of guanidine in the body is still unknown. Burns [1916] showed that its amount increases in the hen's egg during incubation, but no evidence of its source was adduced.

Riesser [1913, 1914] suggested the possible formation of methylguanidine-acetic acid from choline by union with urea according to the equations:

(1) $CH_2(OH) . CH_2 . N(CH_3)_3OH + (NH_2)_2CO$
$= NH_2 . C(NH) . N(CH_3) . CH_2 . CH_2 . OH + 2CH_3 . OH.$

(2) $NH_2 . C(NH) . N(CH_3) . CH_2 . CH_2 . OH + O_2$
$= NH_2 . C(NH) . N(CH_3) . CH_2 . COOH + H_2O.$

By oxidation of the acid, methylguanidine would be yielded.

He adduced evidence that the creatine of muscle is increased after the administration of choline.

Baumann, Hines and Marker [1916] state that on perfusing with choline and urea they found an increase of creatine in the muscles of the dog. Shanks [1921] found that the intravenous administration of choline is followed by a small increase in the creatine of muscle.

Choline occurs in the lecithin molecule in the hen's egg. If it is a precursor of guanidine a decrease in its amount in the egg should occur during incubation while the guanidine is increasing.

Method.

The eggs were weighed and the contents of each ground up with sand. This was then repeatedly extracted with alcohol containing about 10 % of ether. The extract after filtration was made to a definite volume and one-tenth

taken for analysis. Hydrolysis of this portion of extract with excess of $Ba(OH)_2$ in 50 cc. of water was then carried out, boiling under reflux condenser for one hour and filtering while hot. 5 % sulphuric acid solution was then added to precipitate the excess of barium and any excess of the acid was neutralised by the addition of cream of $BaCO_3$. The fluid was then filtered. This filtrate should be perfectly clear. It was then evaporated almost to dryness, dissolved in about 10 cc. of distilled water and filtered. Iodine potassium iodide solution was now added and the estimation of the choline completed as described in a previous publication [Sharpe, 1923].

Six eggs were separately analysed at each period; (i) unincubated, (ii) 7th day, (iii) 14th day and (iv) 21st day of incubation. In some cases the analyses were not completed, but the correspondence between the results was so close that averages for the series analysed at each period are given. They show a steady decrease in the choline content.

Results.

Amounts of choline in the egg of the fowl at different periods of incubation.

Day	Total in g.	%
Unincubated	0·24	0·40
7th	0·21	0·38
14th	0·19	0·32
21st	0·11	0·22

Taking Burns' curve for his sets II to V and expressing it as guanidine it is seen that the rise in the amount of guanidine per egg is from 0·08 g. to 0·26 g., *i.e.* 0·18 g. The decrease in choline found in the present series was from 0·24 g. to 0·11 g., *i.e.* 0·13 g. According to Riesser's equation, 1 g. of choline should yield about 2 g. of guanidine.

These results strongly support the view that choline is a source of guanidine in the animal body.

My thanks are due to Professor Noël Paton for suggesting the investigation and for help and advice during this work. The expenses were defrayed by a grant from the Medical Research Council.

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XXI. THE MODE OF OXIDATION OF FATTY ACIDS WITH BRANCHED CHAINS III. THE FATE IN THE BODY OF α-METHYLCINNAMIC ACID, β-PHENYL-*ISO*-BUTYRIC ACID, AND γ-PHENYL-*ISO*-VALERIC ACID.

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(*Received November 29th, 1923.*)

The work described in the present paper, is a continuation of that previously described [Kay and Raper, 1922].

The enquiry has been extended to phenyl derivatives of fatty acids with a methyl group attached to the α-carbon atom and with a straight chain of three or four carbon atoms.

Although the investigation was undertaken with the hope of finding out the method by which this α-methyl group was oxidised, it has in this particular been unsuccessful. No intermediate products of oxidation which might have thrown light on this process have been discovered, but in other directions, the results are not without interest.

The series of acids investigated up to the present behave on oxidation, in so far as the production of benzoic and phenylacetic acids is concerned, exactly like the similar series described in Knoop's classical experiments on β-oxidation, the only exception being hydratropic acid, which was expected to be, like phenylacetic acid, relatively resistant to attack in the body. Actually as described previously [Kay and Raper, 1922] it was oxidised to a considerable extent, but it did not yield either benzoic or phenylacetic acid as oxidation product.

The results are collected in Table I and with them, for reference, the results of the similar experiments by Knoop with the corresponding straight chain series.

Table I.

α-Methyl series		Straight chain series	
Acid administered	End product	Acid administered	End product
$C_6H_5.CH(CH_3).COOH$	Completely oxidised	$C_6H_5.CH_2.COOH$	Not oxidised
$C_6H_5.CH_2.CH(CH_3).COOH$	Benzoic acid	$C_6H_5.CH_2.CH_2.COOH$	Benzoic acid
$C_6H_5.CH:C(CH_3).COOH$	,,	$C_6H_5.CH:CH.COOH$	,,
$C_6H_5.CH_2.CH_2.CH(CH_3).COOH$	Phenylacetic acid	$C_6H_5.CH_2.CH_2.CH_2.COOH$	Phenylacetic acid

The results on the whole, therefore, bear out the generalisation that acids with branched chains are oxidised in the same way as the parent acids with normal chains. In further support of this statement, another similarity between β-phenylpropionic acid and β-phenyl-α-methylpropionic acid was discovered in the present investigation. Dakin [1909] on administering the former acid to cats, was able to isolate cinnamic acid from the urine, as one of the products of oxidation. In similar conditions, as described in the experimental section, it has been possible to isolate α-methylcinnamic acid as one of the products of the oxidation of β-phenyl-α-methylpropionic acid. This, therefore, provides yet another example of the production of unsaturated from saturated acids in the body.

The results with phenylpropionic acid and phenylmethylpropionic acid just mentioned have another significance which is of interest in connection with the mechanism of oxidation of these acids. It is still unsettled whether direct attack of fatty acids at the β-carbon atom with the formation of β-keto-acids is preceded by the formation of an α–β-unsaturated acid or a β-hydroxy acid. Recent work by Dakin [1923] suggests that the three types of acid—β-keto, β-hydroxy and α–β unsaturated—are in equilibrium in the tissues and that neither the α–β-unsaturated acid nor the β-hydroxy acid can be considered as obligatory stages preceding the formation, by further oxidation, of the β-keto-acid.

The use of the quantitative method, in so far as the experiments will allow, appears to be the only fruitful one at present with which this kind of problem can be attacked, and in the present work, as far as possible, it has been followed.

Although not strictly accurate, because of the type of experiment which has to be used, the results obtained suggest that experiments of this kind are capable of quantitative interpretation where large differences are concerned. In two experiments for instance, in which α-methylcinnamic acid was fed to the same dog, yields of 30 % and 33 % of the expected benzoic acid were obtained. In another dog the yield was 27 %. These results show the order of accuracy that may be obtained. When β-phenyl-α-methylpropionic acid was administered to dogs, the yield of benzoic acid was 77 % of the expected amount.

The authors feel, therefore, that this result indicates clearly that the formation of α-methylcinnamic acid (although it was isolated as an intermediate product) is not an obligatory stage in the oxidation of β-phenyl-α-methylpropionic acid to benzoic acid, for if it were, the yield of benzoic acid, which these two acids give on oxidation in the body, should be approximately the same.

The low yield of benzoic acid from the unsaturated acid suggests that this does not undergo chiefly β-oxidation, but is oxidised with rupture of the aromatic nucleus.

Whether it is a function of the double bond in α-methylcinnamic acid

and other similar substances, to make the aromatic nucleus more susceptible to oxidation, must be left to further experiment.

One other point of interest in connection with α-methylcinnamic acid may be mentioned. As ordinarily prepared, two forms of the acid are obtained which were previously thought to be *cis* and *trans* isomers. According to Stoermer and Voht [1915] these two products are simply isomorphous forms. In solution, they would be expected therefore to show the same chemical behaviour and this was found to be so on submitting them to oxidation in the body. An almost identical yield of benzoic acid was obtained from each. Whether *cis* and *trans* isomers behave alike in the body, has not been determined but it would appear likely that some difference in their ease of oxidation might be expected. The biological evidence tends therefore to support the purely chemical evidence on the nature of these two products.

Experimental Part.

Methylcinnamic acid.

The acid used in these experiments, was prepared partly by Erdmann's method [1885] and partly by Edeleano's method [1887]. Both isomorphous forms of the acid were obtained, the higher melting form (M.P. 82°) was separated by recrystallisation from aqueous alcohol, and was obtained pure after two crystallisations of the crude product.

The mother liquor, containing a mixture of the two forms, was precipitated by evaporating to remove the alcohol and the acid thus obtained crystallised from light petroleum. It was obtained pure, in long prisms, after two recrystallisations and melted at 72°.

For convenience, the two forms are designated *A* (M.P. 82°) and *B* (M.P. 72°).

Form A. The acid was administered to a dog (11 kilos.), as the sodium salt by hypodermic injection, 3 g. of the acid being given in this way on each of two successive days. The urine was collected as usual, from the time of giving the first dose until 48 hours after the last dose had been given.

Distillation in steam of the urine collected during the experiment, did not yield any detectable ketones. The urine was concentrated and after acidifying strongly with syrupy phosphoric acid, was shaken out twice with ether and then extracted for 48 hours with the same solvent in a continuous extraction apparatus. The ether extract, obtained as a residue when the ether was removed by distillation, was extracted several times with warm light petroleum. On removal of the light petroleum, a small amount of acid remained as a crystalline residue. It was converted into the sodium salt and this dissolved in a small volume of water was precipitated with barium chloride. The precipitated barium salts were filtered off and washed with a few cc. of cold water. The acids both from the precipitate and the filtrate plus washings, were recovered by treatment with hydrochloric acid and extraction with ether.

The acid from the barium precipitate was crystallised from light petroleum and was obtained in needles, M.P. 72°; it absorbed bromine and reduced permanganate in the cold and was obviously unchanged α-methylcinnamic acid, but in the *B* form: 0·1 g. was obtained. The acid obtained from the filtrate and washings from the barium precipitation was obtained as a crystalline residue from ether. It was recrystallised from light petroleum, and obtained in glistening plates, M.P. 120–121°. It sublimed on heating and gave a strong smell of ethyl benzoate on warming with ethyl alcohol and strong sulphuric acid, and was therefore benzoic acid: 0·1 g. was obtained.

The bulk of the ether extract obtained from the concentrated urine was insoluble in light petroleum. It was dissolved in hot water, subjected to steam distillation to remove volatile fatty acids, clarified with charcoal and concentrated to about 50 cc. and left to crystallise. 1·93 g. of slightly impure hippuric acid separated and this was obtained pure, M.P. 186–187°, after one crystallisation from hot water (yield 1·67 g.).

The mother liquor contained some substance which reduced permanganate at once in the cold and on warming with the same reagent gave a strong odour of benzaldehyde. The presence of some unchanged α-methylcinnamic acid as the glycine derivative was suspected, and the whole was therefore hydrolysed by adding enough sulphuric acid to make the concentration 20 % and boiling for two hours under a reflux condenser. The solution was extracted several times with light petroleum and the petroleum removed from the combined extracts by distillation. A semi-crystalline residue of acids remained. These were converted into sodium salts, the solution extracted with ether to remove any neutral substances present, and then precipitated with barium chloride, the precipitate being washed with small amounts of water. The barium precipitate and the filtrate plus washings, were treated separately to recover the acids they contained. In each case, hydrochloric acid was added and the solution shaken out several times with ether.

From the barium precipitate 0·12 g. of a crystalline acid was obtained which had all the properties of α-methylcinnamic acid. It crystallised from light petroleum in long needles, M.P. 71–72°, reduced permanganate at once in the cold and decolorised bromine water. On warming with permanganate, it gave a smell of benzaldehyde. The filtrate and washings from the barium precipitate yielded 0·18 g. of benzoic acid, M.P. 121°, which was crystallised from aqueous alcohol.

In this experiment, therefore, of 6 g. of α-methylcinnamic acid administered, 0·22 g. reappeared in the urine, partly free and partly combined (probably with glycine). The rest was oxidised and yielded 1·42 g. of benzoic acid which was excreted almost entirely as hippuric acid. This represents a yield of 30 % of the theoretical (4·35 g.) assuming that the whole of the acid given and not recovered in the urine had undergone β-oxidation to yield benzoic acid.

A second experiment on a dog (8 kilos.) in which 2 g. of the acid were

given on each of two successive days, yielded 1·03 g. of hippuric acid, 0·8 g. of benzoic acid, and 0·1 g. of unchanged acid. This was carried out before the method of isolating the unchanged acid present in combined form in the ether extract was worked out. Possibly a little more unchanged acid was present than the 0·1 g. which was isolated in the free state. The yield of benzoic acid in this experiment was 27 % of the theoretical.

Form B. The procedure used in this experiment was identical with that described in the first experiment with Form *A*. The same dog was used and the dosage was 6 g. of acid as before. The results were almost the same as those given by the acid in its higher melting isomorphous form. A small amount of the acid (0·15 g.) was excreted unchanged in the urine, some of this being free and some probably combined with glycine. The benzoic acid excreted, either in the free state or as hippuric acid, amounted to 1·45 g. This represents a yield of 33 % of the theoretical amount of benzoic acid expected if all the acid had undergone β-oxidation.

The good quantitative agreement between the results of the three experiments with α-methylcinnamic acid, when the methods of extraction and purification of the oxidation products are taken into account, suggests, that the greater part (upwards of 70 %) of the α-methylcinnamic acid, does not undergo β-oxidation to yield benzoic acid, but is oxidised in some other way, which involves rupture of the aromatic nucleus.

Phenyl-iso-*butyric acid* (*β-Phenyl-α-methylpropionic acid*).

The acid was prepared in two ways, (*a*) by the malonic ester method from benzyl chloride and methylmalonic ester, (*b*) by reduction with sodium amalgam of α-methylcinnamic acid. It was purified by distillation under reduced pressure, B.P. 155–156° (11 mm.).

Experiments were carried out with cats and dogs. The general method was the same with both. The acid was given hypodermically as the sodium salt and the urine collected from the first administration until 48 hours after the last dose had been given. The urine was distilled in steam and the distillate examined for ketones by the use of *p*-nitrophenylhydrazine. In no case were any detected although it was expected that acetophenone or propiophenone might be obtained. The urine was now concentrated, and after strongly acidifying with phosphoric acid, extracted with ether. The oxidation products were sought for in the ether extract.

Two experiments were carried out with dogs, and both gave the same result. The second one, carried out quantitatively, was as follows: 3·86 g. of the acid were administered to a dog of 7·5 kilos. It produced no ill effects. The residue from the ether extract of the urine was extracted with light petroleum and from it 0·3 g. of benzoic acid obtained (M.P. 121·5°). The portion of the ether extract which was insoluble in light petroleum was crystallised from hot water and yielded 2·82 g. of pure hippuric acid. No intermediate products of oxidation were detected. The yield of benzoic acid, free and

combined, in this experiment, was 77 % of the theoretical, assuming that β-oxidation alone had taken place. The later experiments were carried out with cats and a larger dose of acid per kilo. was given in order to induce the appearance of intermediate products of oxidation in the urine. This was successfully accomplished in one instance. When the dosage was greater than 1 g. per kilo. the animals died; but with a slightly smaller dose (0·9 g. per kilo.) they recovered after a period of lassitude lasting as a rule for 24 hours. The results of one successful experiment are described. A cat of 2·5 kilos. received two doses of the acid (2 g. and 1·8 g. respectively) a week intervening between the first dose and the second. The urines were distilled in steam in both instances but no ketones were obtained. The residue from the ether extract of the concentrated and acidified urines was repeatedly extracted with warm light petroleum and yielded a small quantity (0·18 g.) of an oily acid, which gave a barium salt soluble in water. It was probably uncharged phenyl-*iso*-butyric acid, but the amount obtained was too small for identification.

The acids from the ether extract which were insoluble in light petroleum were dissolved in hot water, distilled in steam to remove volatile fatty acids, and clarified with charcoal. On cooling, a considerable crop of hippuric acid was obtained. It was recrystallised from hot water and had M.P. 186° (yield, 0·35 g.). The mother liquor reduced permanganate immediately in the cold, suggesting that an unsaturated acid was present. To separate the acids contained in it, the following procedure was used. The mother liquor was neutralised with sodium hydroxide, evaporated to small bulk and precipitated with barium chloride. The precipitate after washing with a little cold water was decomposed with hydrochloric acid and the solution extracted with ether. A small amount of a syrupy acid was obtained which was insoluble in light petroleum but easily soluble in water. It did not reduce permanganate in the cold, but only on warming, when it gave a strong smell of benzaldehyde. This suggested the presence of either β-hydroxy-β-phenyl-*iso*-butyric acid or β-hydroxy-β-phenylpropionic acid, but the amount of acid obtained was too small for further investigation.

The filtrate from the barium precipitate was concentrated to about 5 cc. and then made strongly acid by adding 10 cc. concentrated hydrochloric acid. The solution was boiled under a reflux condenser for two hours to hydrolyse any glycine compounds, diluted and extracted with light petroleum. The extract on evaporation left a colourless, oily, acid residue, which was converted into the sodium salt and precipitated with barium chloride. The acid recovered from the precipitate was colourless and crystalline, and on recrystallising from light petroleum was obtained in needles M.P. 70–71°. 0·1 g. was obtained. It reduced permanganate immediately in the cold, giving a smell of benzaldehyde. After fusion the M.P. rose to 74–76°. This behaviour is characteristic of the form of α-methylcinnamic acid, which when quite pure, melts at 72°, and on melting is partially transformed into the higher

melting variety. A mixed melting point determination confirmed the identity, the two acids after fusing together gave the M.P. 75–77°. The acid recovered from the filtrate of the barium precipitate was small in amount (0·15 g.); it was a viscous liquid, soluble in light petroleum, and was optically inactive. It contained benzoic acid, but the amount was too small to be separated from the liquid component of the mixture. It was therefore not further investigated.

Phenyl-iso-*valeric Acid* (*α-Methyl-γ-phenylbutyric Acid*).

This acid was prepared by the action of phenylethyl chloride on the sodium compound of methylmalonic ester and distillation of the malonic acid derivative. The chief difficulty met with was the preparation of a pure phenylethyl halide. After two unsuccessful attempts by Fittig's method (the chlorination of ethylbenzene, which gives a mixture of chlorides, of which the fraction boiling at 86–87° (21 mm.) contains at least two, probably $C_6H_5.CH_2.CH_2Cl$ and $C_6H_5.CHCl.CH_3$), and an equally unsuccessful attempt by the addition in the cold of hydrobromic acid to freshly distilled styrene (which gives a mixture of the corresponding bromides), Von Braun's method [1911] was employed and a pure ω-chloride was thus obtained. The phenyl-*iso*-valeric acid obtained by the malonic ester method was purified by distillation under reduced pressure and boiled at 169–172° (15 mm.). It was a colourless liquid and did not solidify on standing for several weeks at room temperature. Equivalent, 176; calculated 178. On analysis it gave C, 73·58 %; H, 7·59 %. Calculated, C, 74·11 %; H, 7·91 %. The acid was administered hypodermically as the sodium salt to a cat (2·5 kilos.) on two occasions, 1·2 and 1·5 g. of the acid being given respectively. No ill effects were noted. The urine was collected for 48 hours following each injection, the two portions united, concentrated on the water-bath, and acidified with phosphoric acid. It was extracted with ether containing 10 % alcohol for 48 hours continuously. The residue from the ether extract was purified by distillation in steam to remove volatile acids, clarified with charcoal and evaporated to small bulk. On standing, 0·47 g. of phenaceturic acid crystallised out. On crystallisation from hot water it gave M.P. 143°. A portion hydrolysed with hydrochloric acid, yielded phenylacetic acid, M.P. 77°.

The mother liquor from the phenaceturic acid yielded a further crop of this acid on evaporation, but it was contaminated with a small amount of oily substance. The mother liquor also reduced permanganate in the cold, indicating the presence of unsaturated acids. To isolate the phenylacetic acid, the whole of this crop of crystals and the mother liquor were boiled with strong hydrochloric acid for two hours and the phenylacetic acid extracted with benzene. It was purified by conversion into the lead salt which was crystallised from warm acetone. The acid liberated from the purified lead salt melted at 77°; 0·54 g. was obtained. A search for oxidation products other than phenylacetic acid was fruitless.

The total amount of phenylacetic acid isolated either free or combined

with glycine was thus 0·87 g. or 42 % of the amount to be expected if β-oxidation alone had taken place. The method of isolation was not strictly quantitative but the results suggest that probably not more than half of the phenyl-*iso*-valeric acid yields phenylacetic acid.

Summary.

(1) β-Phenyl-*iso*-butyric acid on oxidation in the body yielded benzoic acid. The yield was 77 % of the theoretical, assuming that β-oxidation alone had taken place. α-Methylcinnamic acid was isolated as an intermediate product of the oxidation.

(2) α-Methylcinnamic acid also yielded benzoic acid on oxidation but no intermediate products of oxidation were isolated. The yield of benzoic acid was only 33 % of that expected if β-oxidation alone had taken place. This suggests that oxidation involving rupture of the aromatic nucleus takes place to a considerable extent.

(3) No difference was discovered in the ease of oxidation in the body of the two isomorphous forms of α-methylcinnamic acid.

(4) γ-Phenyl-*iso*-valeric acid gave rise to phenylacetic acid on oxidation in the body. The yield of this was not more than 50 % of the amount expected if β-oxidation alone had taken place.

(5) The results in general indicate that fatty acids with an α-methyl group undergo oxidation to yield the same products as the parent acids with a straight chain of carbon atoms. No evidence has been obtained which shows whether the α-methyl group is primarily attacked or is removed along with the two end carbon atoms, as in the scheme of β-oxidation suggested by Knoop.

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XXII. THE METABOLISM OF CARBOHYDRATES. PART III.

THE ABSORPTION OF GLUCOSE, FRUCTOSE AND GALACTOSE FROM THE SMALL INTESTINE.

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(*Received December 7th, 1923.*)

In 1919 the author, in collaboration with Pryde [Hewitt and Pryde, 1920], initiated a systematic research on the permeability of the living intestine to various carbohydrates, particularly the biologically important hexoses and their methylated derivatives.

It was hoped in this way to gain some information as to the effect of configuration on the processes of absorption, and as the structural chemistry of the monosaccharides is known with some definiteness, it was thought that the observations would offer some insight into the initial stages of the metabolism of carbohydrates.

The work was abandoned, however, after only a few preliminary experiments, owing to the discovery of optical changes which were interpreted as showing the conversion of the stable α and β isomerides of glucose, fructose and galactose, in part at least, into the highly reactive γ-modifications.

The results of this research were published in 1920 and an extension of the work in 1921 [Hewitt and de Sousa, 1921], while so far the original scheme has not been carried out.

In this paper it is proposed to communicate experiments on the relative rates of absorption of *d*-glucose, *d*-fructose and *d*-galactose from the small intestine.

Previous Work.

The literature on the subject of absorption from the gut is large and, as an excellent digest is given by Goldschmidt [1921], need not be mentioned in detail here.

It may suffice to indicate that, broadly speaking, the views as to the mechanism of absorption fall into two opposed categories, viz.:

(1) There are those who assume that the epithelial lining of the intestine has some special "physiological function" and that the phenomena observed cannot be explained on known physical laws such as those which hold for

diffusion and filtration. Evidence in support of this is given by Cohnheim [1898, 1899], and general agreement with these views is afforded by Reid in a series of papers [1896–1902]. That filtration owing to respective hydrostatic pressures on the two sides of the gut wall is not a determining factor was indicated by Starling [1909], while Höber [1898] and Wallace and Cushny [1898, 1899] showed that solutions of the same osmotic pressure may have differing rates of absorption. Goldschmidt [1921] concluded that the intestine "is a membrane of varying permeability depending upon the nature of the substances presented to it."

(2) Those which postulate a purely mechanical scheme of absorption. The chief upholder of this is Hamburger [1896–1908] who emphasises the effect of intra-intestinal pressure in increasing absorption. Hamburger cites Leubuscher in suggesting that the mechanical unfolding of the folds of the intestine by the contained liquid increases the surface exposed and in this way hastens absorption.

Hamburger and his co-workers [1917–1919] carried out a series of researches in which they investigated the behaviour of isomeric sugars (pentoses, hexoses and disaccharides) towards the glomerular membrane of the kidney of the frog. These authors employed as their criterion of permeability the amounts of each sugar in the urine formed during perfusion with solutions of the various carbohydrates.

If the writer reads Hamburger rightly he attributes the complete retention of glucose by the amphibian renal mechanism to the configuration of the whole glucose molecule and not to the spatial arrangements of the terminal groups only. He suggests that in galactose, one isomeride (which, he does not say) has a molecular architecture adjusted structurally in a manner enabling it to traverse the glomerular epithelium; while with fructose presumably both the α and β isomerides can pass this membrane.

Hewitt and de Souza [1921] investigated the excretion of sugars introduced intravenously into the anaesthetised animal (rabbit) and found no preferential elimination of either α or β galactose, for these and other hexoses when injected as an equilibrated mixture were found in an unchanged condition in the urine.

Present Experiments.

These fall into two groups:

(1) Those in which estimations were made of the amount of glucose or fructose absorbed in various times in different animals from the small intestine. In this set of experiments rabbits were used, and the animals were under full anaesthesia.

(2) Those in which the amount of glucose, fructose and galactose absorbed was estimated in the same animal in the same length of time when making use of the same isolated piece of intestine. In this set of experiments the animals employed were cats.

In view of the work of Osterhout [1916] on the two-fold action of anaesthetics (first a decrease in permeability and second an increase, irreversible and toxic) and of Lillie [1916] who considers that the essential change in anaesthesia is not retarded oxidation but increased stability of cell membranes against permeability, choice was made in this second series of the pithed animal without the administration of anaesthetics during the observations.

General technique.

In all cases the general technique was as follows. The animal was placed under deep narcosis, and, if a cat, was pithed by destruction of the medulla and brain and artificial respiration commenced. The abdomen was then opened in the median line for a short distance, and a suitable convenient length of small intestine carefully withdrawn and isolated by glass cannulae at either end. Through these the loop was thoroughly washed with mammalian salt solution at body temperature until food detritus etc. was removed and the washings no longer reduced Benedict's reagent.

A measured volume of hexose of known concentration was then run in from a pipette, and the solution filling the cannula washed in with a few cc. of salt solution. Clips were then placed on the cannula and the loop replaced in the abdomen which was then closed and kept warm by cloths wrung in saline or by cotton wool.

When the necessary time had elapsed the remaining liquid in the loop was allowed to run into a graduated flask and the loop of intestine was washed with successive quantities of warm salt solution until free from sugar, the whole made to known volume and the hexose content determined by Bertrand's method.

The sugars employed were recrystallised specimens and gave the following constants in aqueous solution:

Sugar		Initial rotation	Final rotation
d-Glucose	2·868 %	103·4°	52·5°
d-Fructose	3·215	−107·8	−92·0
d Galactose	5·60	—	77·0

Solutions of about 2 % of each of the three sugars were made in distilled water at least 12 and usually 24 hours before they were used. By permitting this time interval to elapse between solution and use, any possible complication due to displacement of the usual equilibrium between the α and β hexoses was obviated. In all the experiments reported in this paper the same specimens of sugars were employed.

In addition the following precautions were taken:

(*a*) to introduce only such volume of liquid as would distend the gut to a normal extent; mechanical stretching and abnormal excessive intra-intestinal pressure were thus avoided;

(*b*) to introduce equal volumes of solution in each part of an experiment to keep conditions (as regards pressure) the same throughout the separate observations.

As will be seen below on inspection of the protocols slight differences exist as to the actual concentrations of the solutions employed. These differences, however, are not sufficient to influence materially the osmotic pressure which remains virtually constant and substantially below that of blood.

Immediately before each experiment the three solutions of sugar were standardised against the Bertrand reagents.

Series 1.

Here in 18 experiments involving the use of glucose and fructose, varying lengths of intestine were employed and times of contact of solution and tissue were not kept constant. In addition solutions hyper-, iso- and hypo-tonic were used. As the last (hypo-tonic) comprised 12 of the experiments these only are reported here.

In view of the variables mentioned above, considerable differences in absolute values in individual experiments are perhaps not surprising. It is proposed therefore to give (*a*) the maximum and minimum figures obtained and (*b*) the average absorption of each monosaccharide.

Glucose.

	Loop length cm.	Time minutes	g. introduced	g. recovered
Maximum	48	5	0·261	0·171
Minimum	59	15	0·530	0·470

The amounts absorbed therefore are 34 % and 11·4 % respectively.

Fructose.

Maximum	60	7	0·332	0·255
Minimum	53	8	0·255	0·225

The amounts absorbed therefore are 23·2 % and 11·7 % respectively.

Average of 12 *experiments.*

Glucose. 20 % absorbed in average time of 8 minutes.

Fructose. 16 % absorbed in average time of 7 minutes.

Too great stress is not laid on the above figures in view of the complications outlined and also the condition of narcosis, but it is considered that there are indications that glucose is absorbed more rapidly from hypo-tonic solution than is fructose in approximately equal times and similar concentration when the same amount of sugar is within the lumen of the gut.

Series 2.

In this set of experiments more stringent conditions were attained and at least two, when possible all three, sugars were introduced successively into the same isolated loop. Owing to destruction of brain and medulla under deep anaesthesia no further administration of an anaesthetic was necessary. Ten minutes at least were allowed to elapse between its cessation and the commencement of the observations.

Care was taken to vary the order in which the hexoses were tested, and only those observations were taken as trustworthy in which the fitness of the animal, as judged by the strength and rate of heart beat, was manifest throughout the whole of the experiment.

Control experiments were carried out to determine within what limits recovery of sugar could be effected under the conditions of the experiment and the maximum error obtained was a loss of 10 mgm. sugar, that is 2·6 %.

The protocol of one such control is appended.

1. Cat, died after pithing. All blood vessels to isolated loop were ligatured, the loop washed with 0·9 % sodium chloride as described. When free from reducing substances 20 cm. of 1·68 % glucose were introduced into and retained in the intestine for five minutes. After this it was recovered by salt solution, made up to known volume and estimated volumetrically.

Introduced	375 mgm. glucose	Loss 10 mgm.
Recovered	365 „	

When one remembers that a certain small disappearance of soluble substances may still occur when circulation has ceased, it is seen that recovery can be effected on a basis which is practically quantitative.

Protocols are now given of two experiments where the rate of absorption of all three hexoses was observed when the same piece of intestine was employed.

Emphasis is laid on the fact that in these experiments all conditions were kept constant and equal volumes of salt solution were employed to recover the sugar. In this way the condition of the intestine was kept as far as possible identical before the introduction of each individual sugar.

Experiments are also given where the absorption of two of the three hexoses was estimated under similar conditions.

Sugar in order of examination	Concentration %	g. introduced	g. recovered	Time minutes	Absorbed %
2. Glucose	2·12	0·42	0·21	10	50·0
Fructose	1·87	0·35	0·31	10	10·7
Galactose	2·03	0·41	0·35	10	15·0
		Length of loop 40 cm.			
3. Galactose	1·86	0·38	0·28	10	26·2
Glucose	1·99	0·40	0·24	10	40·0
Fructose	1 86	0·37	0·32	10	14·9
		Length of loop 48 cm.			
4. Glucose	1·90	0·38	0·28	10	26·3
Fructose	1·87	0·37	0·36	10	2·4
		Length of loop 50 cm.			
5. Fructose	1·87	0·28	0·26	10	7·1
Galactose	1·93	0·31	0·23	10	27·5
		Length of loop 37 cm.			
6. Galactose	1·89	0·38	0·26	10	31·2
Fructose	1·86	0·37	0·31	10	17·0

Some time after recovery of the fructose, the heart was noted to be very slow and weak as shown by pulsations in the mesenteric blood vessels. Glucose was then introduced with the following results.

Glucose	1·99	0·40	0·33	10	17·0

Summary of above experiments.

No. of experiment	Amounts of hexose absorbed in 10 minutes		
	Glucose %	Fructose %	Galactose %
2	30	10·7	15
3	40	14·9	26·2
4	26·3	2·4	—
5	—	7·1	27·5
6	(17)[1]	17·0	31·2

[1] See note p. 165.

The salient features of these experiments are:

(*a*) The rapid and large absorption of glucose.

(*b*) The slow absorption of fructose.

(*c*) The intermediate position in this respect occupied by galactose.

(*d*) The individual variations exhibited in different experiments regarding the actual amount of sugar absorbed.

It should be pointed out that the maximum and minimum figures for each hexose are included in the above tables.

In view of the above results, and as a further check of the technique, experiments were carried out in which the epithelial lining of the intestine was impaired. To effect this, two methods were employed: (1) hot salt solution (51°) and (2) dilute sodium fluoride in aqueous solution. The temperature indicated was selected in view of certain work in this laboratory (unpublished) in which it is shown that at or near 47° profound changes occur in living tissues such as blood. The action of sodium fluoride in destroying living tissue is well known, and was first utilised by Reid [1900].

The results of these observations were:

Sugars in order of examination	Concentration %	g. introduced	g. recovered	Time minutes	Absorbed %
7. Fructose	1·86	0·297	0·24	6	19
Glucose	1·99	0·318	0·28	6	15
Intestine washed with 0·9 % sodium chloride at 51°.					
8. Glucose	1·99	0·38	0·34	10	13
Fructose	1·86	0·35	0·31	10	13·6
Galactose	1·87	0·40	0·35	10	12·6
Intestine washed as in (7).					
9. Glucose	1·99	0·40	0·32	10	21
Sodium fluoride run in.					
Glucose	1·99	0·40[1]	0·35	10	12
Fructose	1·86	0·37	0·31	10	17
Galactose	1·89	0·38	0·32	10	17

In this experiment the loop was filled for 3 minutes with the sodium fluoride solution after one observation had been made with glucose.

[1] When the second sample of glucose was introduced the gut was in partial tonus due to the fluoride. A smaller area of membrane therefore was available for absorption.

Summary of above experiments.

No. of experiment	Amounts of hexose absorbed		
	Glucose %	Fructose %	Galactose %
7. Heat	—	19	15
8. „	13·0	13·6	12·6
9. NaF (21)	12·1	17·0	17·0

Discussion and Summary.

The work of numerous observers makes it probable that the mechanism of absorption is the same throughout the whole length of the intestine. Differences undoubtedly exist but these apparently are quantitative only and not qualitative in nature; if this be so it follows that results obtained with the small intestine will hold with but slight differences in other parts of the absorbing alimentary canal.

The experiments reported above show also that the difference between the cat (series 2) and the rabbit (series 1) is not a fundamental one.

Furthermore, it is stated that in hypotonic solutions of diffusible absorbable substances water passes more rapidly than the solute, but that the latter even in dilute solution is also absorbed. This is confirmed as to the absorption of the crystalloid hexoses in the present observations when they are in concentrations with osmotic pressure considerably less than that exerted by blood.

The hexoses employed have similar physical properties, the same molecular weight, the same osmotic pressure, presumably the same rate of diffusion through non-living membranes, and according to Treube [cited from Hamburger, 1904] the same surface tension in solution.

One would, perhaps, thus expect that if absorption was due to osmosis or diffusion alone, with the intestine acting as a dead membrane, all three hexoses would pass through at the same rate.

This does occur when the epithelial lining is destroyed by heat or by toxic agents. Under these conditions the gut appears to act as a permeable membrane and establishes osmotic equilibrium between plasma components and intestinal contents as it does in the case of such substances as magnesium sulphate, sodium sulphate or even lactose, which are absorbed only very slowly.

Starling [1909] notes that monosaccharides are absorbed twice as rapidly as disaccharides, and Bywaters and Short [1913] record glucose as being better absorbed than lactose.

It is however not clear whether the establishment of such osmotic equilibria is entirely due to absorption, in the case of hypotonic glucose, of the solvent, or with hypertonic solutions, of the solute. The possibility of excretion of substances from the blood has to be considered especially in view of the experiments of Fisher and Moore referred to below.

It may be concluded that under the abnormal conditions described the three hexoses are absorbed from the gut at equal rates in hypotonic solution.

With the "normal" intestine a different condition of affairs is experienced and here widely varying rates of absorption exist for the three carbohydrates investigated, the order of rapidity of absorption being (1) glucose, (2) galactose, (3) fructose[1].

[1] Only one discordant result was obtained. Inspection of the notes made at the time reveals no explanation.

It is difficult to attribute the results obtained to any of the following for the reasons given.

(*a*) *Blood flow varying in the experiment.* The duration of the whole experiment was short and those are rejected in which any diminution of the efficiency of the heart was observed.

(*b*) *The order of introduction of the sugars into the intestine.* As already indicated the order was altered as far as possible.

(*c*) *Alteration in intra-intestinal pressure.* The same volumes of solution were run in, in each part of any experiment.

(*d*) *Differing osmotic pressure of the solutions.* The percentage of these varied so little in each experiment that this is virtually constant.

(*e*) *State of metabolism, age, sex, etc. of animal.* Animals were selected promiscuously at various times of the day; the rabbits were not fed for 24 hours immediately before the experiment.

Moore, Roaf and Webster [1911] state that substances in physiological systems will always pass against an osmotic pressure gradient, as the cell contents in the colloidal state possess an attraction for crystalloids.

The question of adsorption of hexoses has been considered by Cohnheim [1913] in connection with their excretion by the kidney. He experimented with kidney pulp and noted differences. Hamburger [1919] criticises the technique and the results obtained are therefore still in doubt. It may be concluded that the conditions obtaining in Cohnheim's experiments are far removed from those holding in the normal glomerular epithelium.

An alternative method of attacking the problem however is available, viz. investigation of resistance of erythrocytes to haemolysis, *i.e.* to alterations of permeability, after exposure to isotonic solutions of the various hexoses. Eisler [1909], by examining the inhibition of specific haemolysis by saponin, has shown that glucose modifies permeability.

The problem in its more extended form is being taken up.

One can note however that the solubilities of the three sugars in lipoidal solvents are not the same but do not vary in the order found for their absorption. The solvent action of lipoids on them is practically nil.

One observation, perhaps, is of importance when viewed in the light of the experiments of Wallace and Cushny [1898, 1899] with equimolecular solutions of inorganic salts and organic esters. These authors found that substances which form an insoluble calcium salt, such as fluorides, oxalates and phosphates, are not appreciably absorbed.

In the experiments reported in this paper, fructose is absorbed most slowly and is the one monosaccharide characterised by the capacity to form a relatively insoluble calcium derivative [Dubrunfaut, 1849, 1856, 1869; Peligot, 1880].

Osterhout [1911, 1915] among others has shown that calcium salts decrease permeability while sodium salts have the reverse action. Lillie [1921] has recently demonstrated this opposed action in star fish eggs. He suggests

that insoluble colloidal salts of calcium are formed with the structural colloids of the protoplasm and are necessary for the normal behaviour of the plasma membrane. In sodium chloride solution water-soluble compounds are formed with disintegration.

In regard to the absorption of glucose, Reid [1900, 1902] proved that glucose in sodium chloride solution was more readily absorbed than in water, while Fisher and Moore [1907] noted that, where a glycosuria exists, there may be no excretion of glucose into the intestine, whereas, if the salt content of the blood is raised by intravenous injection of sodium chloride, this sugar can be found therein.

Permeability may thus be affected from both sides of the mucous membrane of the intestine.

It is not improbable then that union may take place between the hexoses and protoplasmic substance with concomitant variations in permeability. It is indicated that factors other than osmosis and diffusion are at work in the absorption of monosaccharides from the alimentary canal of the normal animal.

While no explanation is forthcoming at present, the results are not without interest when one considers the diet of the young mammal, containing as it does the total carbohydrate supply as lactose which gives rise to the two aldoses most readily absorbed. The necessity for galactose in the formation of the cerebrosides is also significant.

It is commonly stated also that fructose is tolerated to a greater extent than glucose in the diabetic organism. This may well be due to its more gradual and slow absorption compared with glucose.

The whole problem of the metabolism of fructose and galactose is obscure and demands investigation.

Conclusions.

(1) Glucose, fructose and galactose are absorbed from hypotonic solution from the small intestine.

(2) This absorption occurs whether the epithelial lining has been destroyed or not.

(3) When the epithelium has been destroyed all three sugars are absorbed at the same rate.

(4) When the intestinal wall is normal, glucose is absorbed more rapidly than galactose, and the latter more rapidly than fructose.

(5) The results cannot be explained only by osmosis or diffusion.

(6) Attention is drawn to the influence of calcium and sodium salts on the permeability of cells to these crystalloids.

The author is indebted to the Government Grants Committee of the Royal Society for a grant which defrayed the expense incurred.

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ADDENDUM.

Since the foregoing experiments were carried out and the paper written, the author has become aware of the work of Nagano (*Pflüger's Arch.* 1902, **90**, 389–404) in which a research of similar nature is reported.

Nagano employed dogs with a *Vella fistula* and was more occupied with the problem of absorption at different levels of the alimentary canal. His experiments were made with considerable time intervals between each one.

Like the present author he found fructose to be more slowly absorbed than either glucose or galactose, but he concluded that galactose was slightly more rapidly taken up by the dog's intestine than glucose.

Nagano clearly states however that in the dog "the rate of absorption of stereoisomeric sugars is different."

XXIII. LAEVULOSE IN THE BLOOD OF THE HUMAN FOETUS.

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(*Received December 11th, 1923.*)

The presence of sugar in the foetal fluids of herbivora seems to have been first observed by Majewski [1858]. Noël Paton, Watson and Kerr [1907] pointed out that laevulose is the sugar of the amniotic and allantoic fluids of the cow, sheep, pig, cat, dog, rabbit, ferret and guinea-pig, and that it is also present in the blood and in the urine in the bladder of the foetus in the cow and sheep. Takata [1922] showed that laevulose is the sugar of the foetal fluids of the whale *Balaenoptera*.

Sugar is not generally present in the amniotic fluid of the human subject, but Labat and Favreau [1921] state that it is occasionally found in small quantities. It seemed desirable to ascertain if the blood sugar in the human foetus is in whole or in part of the nature of laevulose.

Thanks to the kindness of Dr Hewitt at the Royal Maternity Hospital, Glasgow, specimens of the mixed blood of several foetuses have been collected into alcohol from the placental end of umbilical cords at the time of birth.

Method.

The blood was received in an equal volume of rectified spirit and allowed to stand till the protein precipitate separated leaving a clear supernatant fluid. This was filtered off and the precipitate washed with spirit and then sucked dry. The precipitate was then intimately mixed with more rectified spirit in a mortar and filtered, washed, and sucked dry as before. The precipitate was treated once again in the same manner. The different filtrates and washings were now collected and the alcohol removed almost completely by concentrating under reduced pressure at 40°. The concentrated filtrate was acidified with two or three drops of acetic acid and a slight excess of 20 % normal lead acetate solution added. The precipitate was allowed to settle and the solution filtered, the precipitate being well washed. The excess of lead was now removed by adding a slight excess of dilute sulphuric acid, the requisite amount of alcohol being added to cause complete precipitation of the lead sulphate. The lead sulphate was allowed to settle and was filtered off and washed. The filtrate was then made just acid by neutralising any excess with sodium carbonate. It is important to keep the solution always just to the acid side of neutrality as even in faintly alkaline solution the

glucose tends to change to mannose and laevulose. The solution was now concentrated as far as possible (to about 30 cc.) by evaporation under reduced pressure at from 40° to 45°. The liquid thus obtained was examined for its sugar content by polarimeter and copper reducing value. To detect laevulose the Seliwanoff reaction was used.

Results.

1st Specimen, 26. vi. 22. 300 cc. Foetal blood:
Rotation − 0·03°
Total sugar (by reduction) 0·09 %
Seliwanoff reaction positive.

2nd Specimen, 10. v. 23. 300 cc. Foetal blood:
Rotation + 0·04°
Total sugar (by reduction) 0·075 %
Seliwanoff reaction positive.

The fact that the Seliwanoff reaction was positive in the above cases as well as the abnormally low rotations indicates that *d*-glucose is not the only reducing sugar present in foetal blood. If we assume that *d*-glucose and laevulose are the sugars present, the rotations found compared with the rotations which would have been found had glucose been the only sugar indicate a proportion of *d*-glucose to laevulose of about 2 : 1.

In order to find if laevulose is present in the blood after birth, at least in ruminants, the blood of a kid just after birth was collected in alcohol at the Rowett Institute, Aberdeen, and examined.

3rd Specimen, 10. v. 23. 64 cc. Kid's blood:
Rotation − 0·08°
Total sugar (by reduction) 0·14 %
Seliwanoff reaction positive.

Evidently laevulose does not completely disappear at the time of birth.

For the determination of the rotations I am indebted to Professor T. S. Patterson. The green mercury line was used.

Summary.

1. Human foetal blood has been shown to give the Seliwanoff reaction indicating the presence of laevulose as well as glucose.

2. The persistence of laevulose in the blood of the kid after birth is demonstrated.

My thanks are due to Professor Noël Paton for his help and criticism. The work was done under the auspices of the Medical Research Council.

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XXIV. BLOOD ENZYMES. IV. STUDIES ON THE MALTASE OF DOG'S SERUM: INFLUENCE ON ACTIVITY OF THE REACTION OF THE MEDIUM, AND OF THE STATE OF DIGESTION.

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(*Received December 11th, 1923.*)

In previous communications, data relating to the distribution, the activity, and the influence of the action of heat on the enzyme maltase, as met with in mammalian blood, have been worked out. From the point of view of distribution, these investigations have shown that among mammals there exist two groups: a positive blood maltase group and a negative blood maltase group—man belonging to the latter [Compton, 1921, 2]. As concerns its activity, and its temperature constants (optimum temperature and destruction temperature), the data so far brought to light concern chiefly the enzyme as met with in dog's blood, the dog being a representative animal of the positive blood maltase group.

In the present communication some further questions of importance in connexion with this enzyme are investigated: notably, the influence of the reaction of the medium upon its activity, with a view to ascertaining what is the optimum reaction of the medium for the study of the maltase of dog's blood; what variations, if any, of activity are to be found when the blood under examination is collected respectively in the fasting condition and during digestion; and what respectively is the response of the enzyme to the action of heat in these two conditions.

Influence of the reaction of the medium upon the activity of the enzyme.

This investigation was carried out at the optimum temperature of the enzyme, 55° in an action lasting for 16 hours [Compton, 1922].

The practical details of the experiment were as follows: 0·9 cc. of serum from the dog 2-*P*, in the fasting condition for 24–36 hours, were carefully measured with a pipette into a 10 cc. measuring flask and the volume completed to 10 cc. with pure (redistilled) water. This solution was added in 1 cc. portions to each of six clean sterile test-tubes, containing 90 mg. of pure maltose (hydrated) and either 4 cc. of pure water or of a solution containing

a known quantity of acid or alkali. Five drops of toluene were added and the tubes transferred to a water thermostat regulated at or about 55° (54·8–55·0). After a few minutes, to allow of expansion and escape of vapour while the tubes took up the temperature of the water-bath, they were closed with clean corks which had been washed and sterilised with boiling water. At the end of 16 hours, the enzyme action was stopped by plunging the tubes into boiling water for five minutes, the corks having been previously withdrawn. The tubes were then cooled, the contents diluted to 50 cc. and a Bertrand sugar estimation was made on 20 cc. of the dilution. Fig. 1 shows the results. The serum was seven days old when the experiment was undertaken, this being an interval which is without much appreciable deteriorative effect on the enzyme as met with in the serum.

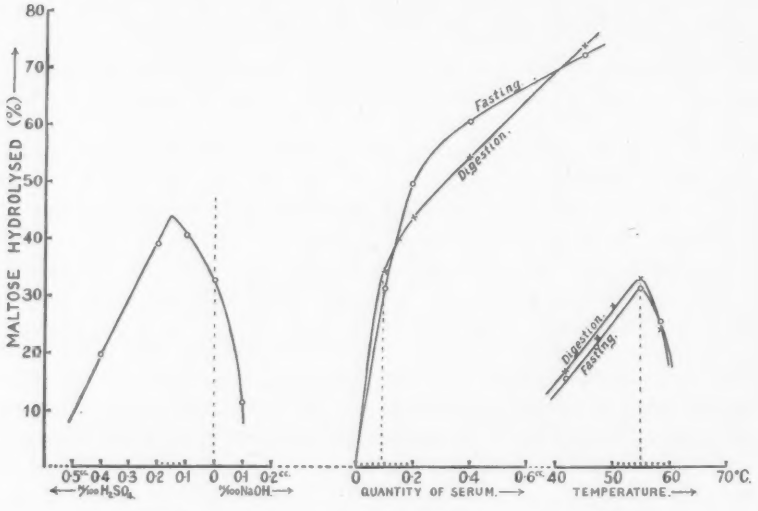

FIG. 1. FIG. 2. FIG. 3.

The curve shown in Fig. 1 indicates that in the conditions of the experiment the merest trace of alkali is detrimental to the enzyme action, while on the contrary the addition of acid increases the efficiency of the enzyme, until a certain point is reached, corresponding to the presence of 0·15 cc. of $N/100$ H_2SO_4 per 0·09 cc. of serum, beyond which the addition of more acid is in turn detrimental to the action. The action, therefore, passes by a maximum situated in the acid region. This optimum works out at 1·66 cc. of added $N/100$ H_2SO_4 per cc. of dog's serum, in a reaction mixture of total volume 55·5 cc.

Hydrogen ion concentration measurements, by the colorimetric method, revealed that this addition of acid to the medium brought about a p_H of approximately 6·6: the natural reaction, without any addition of acid, being 7·4.

It is of interest that this optimum reaction of the medium for a maltase of animal origin is, as regards p_H, practically identical with that found by us for the maltase of vegetable origin met with in *Aspergillus oryzae* [Compton, 1915].

Influence of the state of digestion on the enzyme's activity.

For this investigation, the following comparative experiment was carried out. The dog 2-*P* was allowed to fast during 24–36 hours, previous to the withdrawal of some blood by venous puncture from the jugular vein. It was then fed with a meat meal rich in fat, and 3–4 hours later another specimen of blood was taken. Both specimens of blood were centrifuged as soon as a beginning of clotting was visible. The supernatant serum was pipetted off and conserved in a sterile test-tube in presence of a few drops of toluene. The serum from the digestion specimen was opaque and chylous, owing to the fat partaken of in the meal; the fasting specimen gave a transparent, slightly pink tinted, serum. Parallel experiments were set up with these two freshly collected serums, as follows. Into two series of four test-tubes, were weighed 90 mg. of pure maltose, water was added to each series in quantities of 4·9, 4·8, 4·6 and 4·2 cc., after which the volumes were completed to 5 cc. by the addition respectively of 0·1, 0·2, 0·4 and 0·8 cc. of serum. Five drops of toluene were added to each tube, and they were incubated for 16 hours in a thermostat regulated at approximately 47° (47·5–46·9). The enzyme was then destroyed by heat as previously, and the proportion of maltose hydrolysed in each tube estimated. The experiment gave the result shown by the curves of Fig. 2.

As regards the activity of the two specimens, Fig. 2 brings to light a certain anomaly. It will be seen that, over the range of doses studied, for small doses—for instance, for enzyme concentrations up to 0·1 cc. serum per 5 cc. reaction mixture—the digestion specimen is but slightly more active than the fasting specimen, while for doses between 0·1 and 0·75 cc., the enzyme of the digestion specimen is less active. Beyond 0·75 cc. of serum, the digestion specimen again shows increased activity.

As concerns the activity here revealed by the middle portion of the fasting curve, this is in accord with the findings of Kumagai [1913], who states that the hydrolysis of maltose by dog's serum is a little quicker, and the minimum active dose a little feebler, in the fasting condition than after food. We ourselves in a former study [Compton, 1922] observed in the case of a fresh digestion specimen and a 48 hours old fasting specimen, from the same dog 3-*P*, that the digestion specimen was throughout its course more active in maltase than the fasting specimen; but in this case the latter being somewhat aged the experiment was not deemed conclusive; for it was not certain then if the time the serum had been kept before testing might not be a contributory factor.

Influence of the state of digestion on the optimum temperature of blood maltase.

Two parallel series of temperature studies were carried out 24 hours later with the two foregoing specimens. The quantity of serum employed was 0·09 cc. To measure out this dose conveniently a dilution was made: 1·35 cc. of each of the two specimens was made up with water to 15 cc. in measuring flasks, and 1 cc. of each was distributed respectively into two series of six test-tubes, containing 90 mg. of weighed maltose dissolved in 4 cc. of water and five drops of toluene. The tubes were incubated in six thermostats regulated at temperatures varying between 42° and 58°. After 16 hours the enzyme action was stopped by placing the tubes in boiling water. On estimating by Bertrand's method the proportion of maltose hydrolysed, the two series gave the results shown in Fig. 3.

This figure shows that the optimum temperature of the maltase of dog's blood is the same—55° for the time interval and the natural reaction of the medium—whether the specimen be taken when the dog is fasting or in digestion.

This finding is of special interest, in view of the fact that the optimum temperature of an enzyme is very sensitive to any p_H alteration of the medium used to determine it [Compton, 1915; 1921, 1]. It affirms the constancy of the p_H reaction of the blood—at least of the venous blood—of the individual; that this does not vary but remains constant even during digestion, when a variety of substances enter the blood stream from the breakdown by alimentary digestion of the complex molecules of food.

The result indicated by this study possesses the more interest for us in that we had already observed in previous work [Compton, 1922] under similar experimental conditions, at least of duration and of concentrations in enzyme and substrate, that the optimum temperature of the enzyme as met with in the blood of different dogs (2-*P*, 3-*P* and 4-*P*) was constant at 55°, in an action lasting for 16 hours whether the serum of the animals was tested fresh or a few days old; and also, in the case of one of the dogs (3-*P*), when the optimum temperature was determined on one occasion with a fresh digestion specimen, and on another with a fasting specimen two days old.

Now, enzyme preparations on ageing often show considerable loss of activity, especially when in aqueous solution; also, when preserved in the condition of a dry powder, they undergo transformations such as are revealed by changes in the optimum p_H reaction which they will support [Bertrand and Compton, 1921]. In the comparative experiment on optimum temperature described in this paper, by utilising specimens of blood withdrawn from the same dog and tested on the same day in exactly the same fashion, the experimental factors were rendered so nearly identical that any possible alterations of the enzyme which might result from ageing of the serum were reduced to a minimum and practically eliminated.

Our experiments of an earlier date and those of to-day, in the constancy of optimum temperature which they portray, cannot well be interpreted otherwise than in the fixity of the chemical, or p_H, reaction of the blood itself. They are but expressions of the same. The buffering of the blood is so perfect for the species that from individual to individual the optimum temperature of any contained ferments does not vary.

SUMMARY.

Optimum reaction of the medium for the maltase of dog's blood. In an action lasting for 16 hours at 55° (optimum temperature under these conditions for the natural reaction) in a substrate concentration of $M/20$ and an enzyme concentration of 0·09 cc. per 5 cc. reaction mixture, the optimum reaction is reached by the addition of 0·15 cc. of $N/100$ H_2SO_4.

In respect of the enzyme requiring an acid medium in which to act to best advantage, no distinction is to be drawn between maltase of animal and maltase of vegetable origin.

State of digestion and activity of the enzyme. For all practical purposes the activity of the enzyme is the same whether the blood be collected during digestion or in the fasting state, only very slight differences being recorded, such as might be expected in oscillations about normal limits.

State of digestion and optimum temperature of the enzyme. The optimum temperature is unaffected by the state of digestion of the animal at the time the blood is withdrawn for investigation (whether fasting, or immediately after a meal), being 55° in an action lasting for 16 hours for an enzyme concentration of 0·09 cc. per 5 cc. reaction mixture.

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XXV. THE RELATION OF THE VITAMIN A POTENCY OF THE LIVER OIL TO THE SEXUAL CONDITION AND AGE OF THE COD.

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(*Received December 17th, 1923.*)

It was previously pointed out [Zilva and Drummond, 1922] that a very appreciable disparity exists in the vitamin A potency of cod liver oils. This variation in potency we have shown could not be due to the various modes employed in the preparation of the oils [Drummond and Zilva, 1922]. It must, therefore, be traced to some biological factor. The biological causes which suggested themselves as being likely to influence the potency of the oils were the sexual condition, the age, and the food of the fish. We are now able to bring forward evidence showing that the first two factors do not influence the activity of the oil to any marked extent.

The Potency of Oils from Fishes in Different Stages of the Reproductive Cycle.

It is well established [Hjort, 1914] that the fat of the whole system of the cod, especially of the liver, is being utilised in the process of development of the genital products during spawning. This transmission of fat was expected to be also attended by a transmission of the vitamin, a supposition which was strengthened by the experimental evidence of Coward and Drummond [1922] who demonstrated the part played by the vitamin in the development of the larval trout. This passage of the vitamin to the genitals might either have had no influence on the vitamin concentration of the residual oil of the liver in spite of the diminution of the oil content of the organ or it might have removed more of the vitamin in proportion to the oil. In the former case the potency of the oil during the various stages of the reproductive cycle would not be affected appreciably, in the latter case the livers would yield oils with a diminished vitamin content during the spawning period.

After studying a great number of cod in various stages of their reproductive cycle we have adopted the following standards for their sexual condition:

Males.

"Narrow"	Convolutions of spermatogenic tissue		Immature
"Medium"	,,	,,	Mature fish, successive stages of ripening testis
"Broad"	,,	,,	
"Opaque"	,,	,,	
"Running"	,,	,,	

Females.

White	Undeveloped	Stages of gonads in immature fish, the succession of which is uncertain
Cherry	,,	
Orange	,,	
Vermilion	Developed	Mature fish, successive stages of ripening ovaries
Orange	,,	
Yellow	,,	
Cream	,,	
Mottled	,,	
Running	,,	

Further details of this differentiation are given by Graham [1923].

The livers and gonads were taken from "live" cod at Flamborough and Scarborough, *i.e.* cod which have been caught by lines so recently that gutting has not been necessary. The livers and gonads were removed immediately on landing and despatched on ice to London. The organs reached the Lister Institute, where the manipulations were immediately proceeded with, not later than 18 hours after the fish had been caught. When the tissues arrived at the laboratory they were immediately weighed, the gonads were preserved in a known quantity of alcohol and the livers were melted out in a steam-jacketed pan. The oils thus obtained and the gonads were then tested out on rats for their vitamin A content by the Zilva and Miura [1921] method. These experiments were carried out on four occasions in 1922, namely, January and

Table I.

Date	Sex and condition of reproductive organs	Minimum growth-promoting dose in mg.
23. i. 22	♀ "Cherry undeveloped"	1·8
	♀ "Orange"	1·8
	♂ "Narrow"	1·4
	♂ "Broad"	1·8
21. iii 22	♀ "Cream"	1·4
	♀ "Mottled"	1·4
	♂ "Opaque"	Less than 10
25. iii. 22	♀ "Spent"	1·8
	♂ "Spent"	1·4
vi. 22	♀ "Spent"	2·2
	♂ "Spent"	2·2

March utilising unspent fish, and March and June when spent cod were investigated. As will be seen from Table I liver oils from fish of both sexes in different conditions of their reproductive period were studied. Little variation in the potency of the oils could be recorded. Even the liver oils from spent cod in March fish, which must have spawned just before being caught and which had no time to recuperate, did not deviate in potency from the oils of fish before spawning and several months after spawning and consequently after recuperation.

The preserved gonads, after removal of the alcohol in a vacuum at a temperature of about 40°–50° were also tested out for vitamin A and were found to be very potent (minimum growth-promoting dose 0·025 g. in the case of the male, and 0·05 g. in the case of the female, of fresh roe). It is, therefore, evident that as the spawning period approaches the oil and the vitamin from the liver both pass into the gonads. The relative potency of the residual liver oil is, however, not affected.

Further confirmation of this observation that the fluctuation in the potency of liver oils is not controlled by the condition of the fish in the reproductive cycle was obtained by examining liver oils from various fishes caught in the North Sea and in the vicinity of the British Isles at different times of the year, namely, September, 1921 and 1922, November and December, 1921, January, February, April, May, June, July, August in 1922. During this period no serious fluctuation could be observed in spite of the number of samples (about 40 in number) that were examined. We wish to take this opportunity of expressing our very best thanks to Messrs Isaac Spencer and Co. of Aberdeen, and Mr Fairbank Kirby, of Grimsby, through whose kindness we were able to secure all these oils.

The Potency of Oils from Fishes of Different Ages.

This problem was investigated in Newfoundland on two supposed races of cod; one occurring south of St John's (Ferryland) and another north of it (Port Union and Winterton). The fishes were measured for length, gutted and their livers were steamed out under comparable conditions within a few hours of capture. They were then classed according to age, utilising the length and the condition of the development of the sexual organs as criteria. Scales were also taken but the scale readings were inconclusive and the results could not be utilised for age determination. Table II gives the details of the fishes

Table II. *Cod.*

Ferryland

		No. of fish	Mean length cm.	Range cm.
♂♀	Immature	59	44·1	37–51
♂♀	„	44	44·2	39–53
♂♀	Mature	23	77·9	65–97
♂♀	„	9	75·3	67–85

Difference between Means of Immature and Mature: ♂ 33·8 cm.; ♀ 31·1 cm.
Gap between Ranges ... ... ♂ 14 „ ; ♀ 14 „

Port Union and Winterton

		No. of fish	Mean length cm.	Range cm.
♂♀	Immature	47	36·5	27–47
♂♀	„	50	37·3	26–47
♂♀	Mature	21	74·4	64–85
♂♀	„	13	73·9	67–79

Difference between Means of Immature and Mature: ♂ 37·9 cm.; ♀ 36·6 cm.
Gap between Ranges ... ... ♂ 17 „ ; ♀ 20 „

employed. As will be seen from the table the gap in length between the mature and immature groups is 14 cm. in the southern variety and 17 to 20 cm. for male and female respectively in the northern variety. Although the exact rate of growth of the cod is unknown it is nevertheless certain that 14 cm. represents *at least* one year difference. We have thus been able to classify them according to age, maturity and to sex. The livers of the respective groups were pooled for the preparation of the oils. The samples of oil were bottled, care having been taken to exclude air, and despatched to England and were tested out immediately on their arrival at the Lister Institute. These oils have been found to be very potent [Zilva and Drummond, 1923] (minimum growth-promoting dose 1·6 mg.) but no difference was established between the oils of the various batches. The fluctuation in the potency of liver oils cannot, therefore, be attributed to the preponderance of fishes of a certain age at different times.

The above experiments make it plain that neither the sexual condition nor the age of the fish can be responsible for the less potent oils sometimes obtained from livers of *certain* batches and one is forced to turn one's attention to the food. We [Drummond and Zilva, 1922] have shown that the ultimate origin of vitamin A must be traced to unicellular marine organisms. It is not directly from these organisms that the cod receives its vitamin but through several intermediaries such as the copepods and larval decapods and mollusca which are present in plankton and which feed on these unicellular plants. These are in turn consumed by the capelan and other forms of food of the cod. One may, therefore, assume that insufficient synthesis of the vitamin by the unicellular marine organisms owing to adverse natural conditions would ultimately be reflected on the vitamin content of the liver and gonads of the cod and would most probably also influence the mortality of the larval cod. The problem is of great interest especially to the marine biologist and invites further investigation.

SUMMARY.

The sexual condition and age of the cod do not influence the vitamin A potency of the liver oil.

The expenses of this enquiry were defrayed by grants from the Medical Research Council, including a whole time grant to one of us (S. S. Z.), the Newfoundland Government and the Newfoundland Board of Trade. Our thanks are due to the above bodies.

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XXVI. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. I.

By SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute, London.

(*Received December 31st, 1923.*)

It has been shown [Zilva, 1923] that on treating decitrated lemon juice with alkali, inactivation of the antiscorbutic factor is brought about in the presence of air with a simultaneous increase in the hydrogen ion concentration of the solution. In the absence of air this treatment with alkali does not influence either the activity or the reaction to any perceptible degree. The increase in the hydrogen ion concentration was attributed to the probable formation of acid from the sugar in the solution. It was also pointed out at the time that the destruction of the vitamin proceeded at a much quicker rate than the formation of the acid, an observation which excluded the probability of a direct connection between the two changes. The results did not, however, exclude the possibility of a connection between the formation of an intermediate compound in the production of the acid and the inactivation of the vitamin. The investigation was, therefore, followed up on these lines with the ultimate object of obtaining more information on the chemical character of the antiscorbutic factor.

It was desirable at this stage to ascertain whether the sugar present in active decitrated lemon juice has any bearing on its antiscorbutic activity and the obvious task was, therefore, to remove the sugar and see whether the potency was destroyed. The removal of the sugar from the active solution without actually impairing the rather unstable antiscorbutic factor was found to be associated with certain difficulties. Owing, however, to the recently ascertained facts concerning the conditions of the inactivation of the antiscorbutic vitamin it was possible to devise a biochemical method of removing the sugar without perceptibly influencing the antiscorbutic activity, namely, by fermenting the sugar with yeast in an atmosphere of carbon dioxide.

By this method it is possible to eliminate almost the entire sugar content without perceptibly influencing the activity of the solution. This observation disposes of the idea that any connection exists between the oxidation of the sugar and the inactivation of the antiscorbutic factor in decitrated lemon juice. Furthermore, evidence can now be produced showing that the antiscorbutic factor in the fermented decitrated lemon juice behaves in alkaline

solution in the presence of air similarly to that in unfermented decitrated solutions.

The elimination of the sugar from the decitrated lemon juice without influencing its activity limits further our field for the chemical investigation of the potent fraction. We may now exclude from the total solids of lemon juice the organic acids which form the bulk of the solids and the sugar as having no connection with the activity. The average dry matter content of decitrated lemon juice is about 1 % (calculated on the original volume of the lemon juice) although samples with a content as high as 1·7 % of solids are occasionally observed. When the sugar which is shown to be invert sugar is deducted from this figure it leaves us with an active fraction containing less than half of the dry matter of the decitrated juice. This has a nitrogen content varying from 0·004 %–0·014 %. The residual active fraction consists mostly if not almost entirely of nitrogenous substances. Although the investigation of the chemical character of these nitrogenous substances is not yet complete a certain amount of preliminary information has been obtained at this stage and is given in another part of this communication.

Experimental.

On preparing an osazone from decitrated lemon juice, glucosazone is obtained. Microscopical examination of the crystals shows that no other osazone is present. The decitrated lemon juice also gives a very marked Selivanoff reaction. The solution is laevorotatory and reduces Fehling's solution. In assuming that the sugar present is entirely composed of invert sugar and calculating accordingly, the sugar content from the reduction values obtained by Bertrand's method, a specific rotation is obtained which approximates that of the supposed sugar. The following specific rotations were observed in five typical experiments: − 19°, − 17·5°, − 16·5°, − 22·9°, − 16·5°, giving an average $(\alpha)\frac{D}{18}$ − 18·5°. The theoretical value for invert sugar is − 20·1°. These facts show that the sugar in decitrated lemon juice consists mostly, if not entirely, of invert sugar.

In order to remove the sugar by fermentation precautions were taken to ensure an acid medium and the absence of air. In the early experiments the acidity was regulated by the addition of succinic acid. It was found later, however, that fermentation in an atmosphere of carbon dioxide gave equally good results. The following procedure was adopted. To 50 cc. of decitrated lemon juice 0·5 g. of succinic acid and 1 g. of washed brewer's yeast were added. Carbon dioxide was then passed for about 15 minutes, and the flasks closed with a rubber stopper containing a mercury seal. The mixture was incubated for about 18 hours at 25° at the end of which time it was filtered through a Berkefeld filter. The solution thus obtained was ready for feeding purposes and had a hydrogen ion concentration of about p_H 3. The yeast used in these experiments was obtained fresh from the brewery on the day

when the fermentation was carried out. It was pressed free of wort, washed and centrifuged once with tap and once with distilled water and filtered on a Buchner funnel, until the residue became a doughy mass. The solutions were fermented every other day and were administered on the two following days, being stored during the interval in the absence of air at room temperature.

To control the above fermented solution, decitrated lemon juice containing the same quantity of succinic acid was incubated for the same time at 25° without yeast, and another batch of juice of the same acidity was treated with the same quantity of yeast as the above only for 15 minutes, filtered after that time through a Berkefeld filter and incubated at 25° for 18 hours. This last experiment was instituted with the purpose of eliminating any error that might have been produced by adsorption by the yeast. The reduction of the solutions before and after treatment was determined in all the batches by Bertrand's method. It was found that in all the controls there was hardly any change in the reducing power of the solutions whilst in the fermented solution almost the entire reducing sugar was removed by the treatment. (About 0·2 cc. of Bertrand $KMnO_4$ required for 2 cc. of fermented solution.)

The three batches were tested out for their antiscorbutic content on guinea-pigs. As will be seen from Table I the decitrated lemon juice from which the invert sugar has been removed by fermentation showed as good an activity as that of the control batches.

Table I.

Treatment of the decitrated lemon juice	Daily dose in cc.	No. of days animal lived	Cause of death	Degree of scurvy
Fermented	1·5	60	Chloroformed	+
	1·5	57	Cause unknown, not scurvy	+
	3	60	Chloroformed	Nil
	3	60	,,	+
	5	60	,,	Nil
	5	60	,,	,,
Incubated without yeast ...	1·5	43	Cause of death not acute scurvy	+ +
	3	60	Chloroformed	+
	5	57	Cause of death not scurvy	Nil
Treated with yeast for 15 mins	1·5	50	Scurvy	+ + +
	3	60	Chloroformed	+
	5	60	,,	Nil

A similar retention of the antiscorbutic potency by fermented decitrated lemon juice saturated with CO_2 in the absence of air was shown by the following experiment. The juice was treated with the same quantity of yeast under the same conditions as in the previous set of experiments only no succinic acid was added. After fermentation and filtration through a Berkefeld filter the preparations were fed to guinea-pigs in daily doses of 1·5 cc., 3 cc. and 5 cc. All the animals (two on each dose) survived the period of two months after which time they were chloroformed. With the exception of those which received the daily dose of 1·5 cc., which showed very slight signs of scurvy, all the animals were found to be normal. These results are identical with those

obtained with unfermented decitrated lemon juice and, therefore, the antiscorbutic is not influenced to any marked degree by the removal of the invert sugar by fermentation.

It was further found that the activity of fermented decitrated solutions is influenced by reaction and the presence of air as in the case of the unfermented solutions, namely, that in the absence of air $N/20$ alkali had no marked deteriorating action on the antiscorbutic potency whilst a solution of the same reaction had an inactivating influence when no precaution was taken to exclude air. The technique employed in these experiments was the same as that already described [Zilva, 1923]. A daily dose of 5 cc. of the fermented solution made $N/20$ alkaline and kept under laboratory conditions for 24 hours failed even to delay the onset of scurvy in guinea-pigs whilst a dose of 1·5 cc. of such a preparation kept for the same time under strictly anaerobic conditions maintained the animals for 56 days.

The above experiments bring us a step further in the investigation of the active fraction of lemon juice and afford the possibility of obtaining an antiscorbutic more free from impurities than any potent substance so far described. As already mentioned, indications which are so far available seem to show that the active fraction is a complex mixture of substances, the better part of which are of a nitrogenous character. Fermented decitrated juices give negative biuret, sulphur and tryptophan tests. A very faint murexide reaction which is masked by a brown coloration is generally obtained. Precipitates are obtained with mercuric sulphate, and basic lead acetate. Phosphotungstic acid occasionally, but not invariably, gives a very slight precipitate. Millon's reagent gives a precipitate, which is soluble in excess of the reagent. The solution reduces ammoniacal silver nitrate and gives a marked Pauli reaction. No amide nitrogen can be detected. Very little extraneous matter seems to be introduced by solution from the yeast and most of the above reactions can also be obtained in the unfermented decitrated lemon juice. It is hoped that a detailed investigation of this fraction will afford further information on the chemical nature of the antiscorbutic vitamin.

Summary.

A great part of the solids of decitrated lemon juice consists of invert sugar. It is possible to ferment the sugar and thus reduce the solids without appreciably altering the antiscorbutic activity of the solution. The remaining solids seem to consist of substances mainly of a nitrogenous character. Some chemical characteristics of the active fraction are given.

The author wishes to express his thanks to the Medical Research Council for a whole time grant.

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XXVII. A NOTE ON THE CONSERVATION OF THE POTENCY OF CONCENTRATED ANTISCORBUTIC PREPARATIONS. II.

By SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute, London.

(*Received December 31st, 1923.*)

It was previously found [Zilva, 1923] that if concentrated decitrated lemon juice is stored in acid solution in a vacuum over pyrogallic acid no demonstrable loss in the antiscorbutic potency takes place within three months, thus demonstrating that by observing conditions which prevent the oxidation of the vitamin it is possible to keep concentrated active solutions for some time. The investigation was extended with the purpose of applying this principle to a method which would render possible the preparation of such concentrated solutions for practical application.

Decitrated lemon juice was, therefore, prepared as described in the previous note, concentrated ten times, and acidified (7 g. of citric acid to the litre of juice). 50 cc. of this concentrated juice was then introduced into each of a series of flasks which were tightly stoppered with a rubber stopper containing a thick-walled capillary, evacuated and sealed. The flasks were stored under laboratory conditions in a dark cupboard. Batches were opened three and five months after the preparation and tested out on guinea-pigs. As was done before, the juice was diluted to its original volume and stored in a vacuum over pyrogallic acid during the period of testing out. No loss in potency could be established at either period. The equivalent of 1·5 cc. of the original juice immediately after preparation and after storage was found to be the minimum daily dose required to maintain a guinea-pig on a scorbutic diet for two months without the onset of scurvy.

Clinical confirmation of the antiscorbutic potency of this stored preparation was obtained by utilising it in the treatment of a case of infantile scurvy after six months' storage. Through the kindness of Dr G. F. Still I am able to give the clinical notes of the case which was treated in one of his wards at the Hospital for Sick Children, Great Ormond Street.

Monica, T. Aged 8 months. Admitted October 30th.

Admitted for loss of power in legs and to a less degree in arms. Six weeks ago cried when bathed and lost power in legs, recently seems to be tender in arms.

Has been fed on Allen and Hanbury's Food, No. 1, since birth, with addition of Virol during past three months, and Bovril during last two months. Has recently had some boiled egg on alternate days. During last 10 days has had baked apple, one teaspoonful 2 times, and stewed prunes 3 times. Has had half a teaspoonful grape juice 3 times in last week. No previous illness.

Present Condition.

Fairly nourished, lies quiet when not touched, but cries when touched or moved. Lies with legs almost motionless, slight movement of toes and ankles. There is some thickening about the left femur especially at the upper part. There is slight thickening just above both radial epiphyses.

Two incisors present in each jaw, gums only slightly purplish, not typical on admission.

Urine 3 days after admission showed a few blood corpuscles. Arms lie flaccid at side but some movements of wrists and fingers. Very marked scorbutic pseudo-beading of ribs.

At 5.30 p.m. on October 30th concentrated decitrated lemon juice begun, 2½ drachms given and repeated at 11 p.m.

At 7 a.m. next morning nurse reports decidedly less tender and no pain on being washed.

During October 31st concentrated lemon juice continued.

8 a.m.	... ...	2½ drachms
11 a.m.	... ...	2½ „
2 p.m.	... ...	6 „
5 p.m.	... ...	4 „
8 p.m.	... ...	3 „

At 9 a.m. on October 31st more movement of legs and arms noticed and at 6 p.m. definitely improved, flexing left leg readily at knee and right leg also to less extent, both arms moved much more freely, will play with toy, does not object now to being handled.

Stool becoming unhealthy, green with mucus.

November 1st. Stool loose, offensive with mucus. Improvement in limbs continues.

Nov. 1st	8 a.m. ...	3 drachms	Nov. 2nd	5 a.m. ...	1 drachm
	11 a.m. ...	3 „		11 a.m. ...	1 „
	2 p.m. ...	3 „		5 p.m. ...	1 „
	5 p.m. ...	3 „		11 p.m. ...	1 „
	11 p.m. ...	1 drachm	Nov. 3rd	5 p.m. only	1 „

November 2nd and 3rd amount of concentrated lemon juice had to be diminished as stools continued bad, mucus being passed in considerable quantity and the number of stools 3 and 4 per diem.

By November 4th movement of arms was quite free and by November 6th all limbs were moved normally. Stools continued to show mucus but gradually improved and were pale but otherwise normal by November 8th. On and after November 4th 1 drachm of the concentrated lemon juice was given at 5 a.m. and 5 p.m. until 5 a.m. on November 9th, when it was discontinued as the child was discharged well that day.

It will be seen that the treatment was very intensive. One drachm of the concentrated juice corresponds approximately to the antiscorbutic content of the juice of one lemon and consequently the equivalent of about 48 lemons was administered during the treatment and the equivalent of about 15 lemons was given to the child within the first 24 hours. This, of course, would have been out of the question with any natural active juice. The rapid recovery of the patient confirms the laboratory observations made on this stored preparation.

The above experiments establish the conditions under which a very active antiscorbutic preparation can be stored without deterioration for months under conditions suitable for practical applications and we are thus in a position now to utilise this very potent antiscorbutic in the treatment and prophylaxis of adult and infantile scurvy. It is necessary to observe that the citric acid added was somewhat in excess, as it was originally intended to test the preparation clinically as a prophylactic in which case it would have been used in a diluted condition. It is anticipated that decitrated lemon juice from which the invert sugar has been removed by fermentation in an atmosphere of CO_2 as described in the preceding communication [Zilva, 1924] could also be stored without loss of potency. This would afford an antiscorbutic with even less extraneous matter. Experiments on these lines are in progress.

The author wishes to express his thanks to the Medical Research Council for a whole time grant.

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XXVIII. A SIMPLE AUTOMATIC PIPETTE.

By GEOFFREY ARTHUR HARRISON.

From the Biochemical Laboratory, King's College Hospital.

The apparatus figured was devised originally for use in repeated estimations of blood sugar. It has been so valuable and could be adapted for so many purposes, that it is presented here in the hope that it will prove of assistance to other workers.

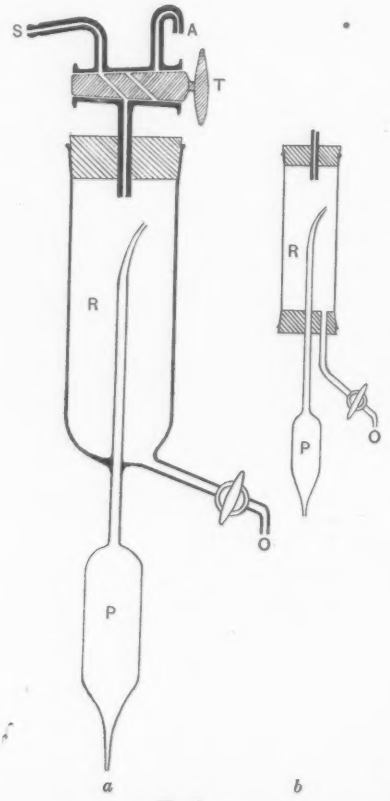


Fig. 1.

The pipette *P* is calibrated to deliver exactly the required volume in the usual way, any excess of fluid adhering to the exterior being removed by contact with another vessel both before and after delivery. The pipette is fixed into the reservoir *R* by means of an india-rubber cork (Fig. 1 *b*), or where rubber is not permissible it may be fused in (Fig. 1 *a*). *O* is a side tube with glass tap through which the overflow may be run off or returned to stock as desired. The pipette is filled by suction with the solution, a few hundred cc. of which may conveniently be kept in a wide-mouthed, small stoppered bottle.

T is a two-way tap fixed through the upper cork in the reservoir. When open to the left, *S*, fluid is sucked into the pipette, and overflows into the reservoir, *R*. When this has occurred the tap *T* is turned through 90°, thereby shutting off the suction *S* and the air inlet *A*. The end of the pipette is kept just under the surface of the fluid until there is no further overflow. The solution is then removed, when it will be seen that the contents of the pipette remain stationary for a period of time ample to allow of placing the receiving vessel in readiness. At the most there will be a barely perceptible movement of the capillary meniscus at the upper end of the pipette. The contents of the pipette are delivered by turning the tap *T* to air by a further rotation through 90° in the same direction as before.

I have tested various sizes of pipette from 1 to 25 cc., and have found them all equally satisfactory. Three or four pipettes could be fixed into the one reservoir if desired, particularly in dealing with small volumes.

A battery of automatic pipettes may be fitted in series to the one suction pump with the aid of *T*-pieces. It is useful to interpose between the suction pump and the apparatus a filter flask fitted with a capillary by-pass, so that the negative pressure in the automatic pipette never becomes great enough to cause too sudden a rush of fluid.

The following advantages are claimed for this automatic pipette. It is very simple and accurate, even more accurate than an ordinary pipette. There is no mark to watch when filling it. All contact of the solution with rubber or with grease may be avoided. It may be cleaned and dried *in situ* by running through water and then acetone, or by other similar methods.

The apparatus described is virtually a modification of Wright's automatic capillary pipette.

XXIX. BACTERICIDAL ACTION OF THE TELLURIUM DERIVATIVES OF ALIPHATIC β-DIKETONES. PART II.

By GILBERT THOMAS MORGAN, EVELYN ASHLEY COOPER and ARNOLD WIGHAM BURTT.

From the Chemical Department, University of Birmingham.

(*Received December 21st, 1923.*)

Although the β-diketones are feeble germicides [Morgan and Cooper, 1921], their tellurium derivatives recently discovered by Morgan, Drew and their co-workers [1920, 1922] contain this metalloid in the unsaturated bivalent condition and are accordingly likely to exhibit stronger germicidal action in view of the circumstance that the more saturated telluric acid is known to possess antiseptic properties. Preliminary experiments on four of the tellurium derivatives by the Chick-Martin test revealed high phenol coefficients, but it was found that further growths occurred on subculturing the broth tubes into fresh broth. It was accordingly suggested that these compounds were not true germicides, but rather growth inhibitors, and it was calculated that tellurium propionylacetone would exert a definite inhibitory effect in a concentration of 1 in 5,000,000.

In a subsequent paper [Morgan, Cooper and Burtt, 1923] the inhibitory method showed that these compounds were inhibitors to micro-organisms in remarkably low concentrations, for instance, tellurium dipropionylmethane in 1 in 10,000,000 to 1 in 40,000,000. An examination of several compounds of this type indicated that the bactericidal power increased considerably as the homologous series was ascended, until with a second substitution of methyl the maximum efficacy was attained, and beyond this limit there was a diminution in germicidal power. Moreover, it was noticed that the chemical structure of the parent β-diketone influenced bactericidal power, and that position isomerism was also an important controlling factor.

Many other compounds of this type have since been prepared and examined especially with the view of ascertaining whether they are suitable for purposes of inner disinfection.

Experimental Method.

Preliminary experiments showed that the Chick-Martin test was unsuited to the examination of these compounds and in the present work the inhibitory test was used exclusively. The method has been described and discussed in a previous paper [1923], the inhibitory action of the tellurium compounds being such that growths frequently became apparent on the second day only and the tubes were generally incubated for three days or more. Tubes free

from visible growth at the end of an experiment have been subcultured into fresh broth, but in no instance has further growth occurred. It is evident, therefore, that while these compounds belong to the "inhibitor" class they are nevertheless true germicides, differing from those of the phenol type only in that they act very slowly and in exceedingly low concentration. Since some of the compounds examined were of doubtful stability, freshly made solutions were always employed and the substances dissolved in water without undue heating. Lemco-peptone broth (acidity $+7E$) was used as a standard medium, but for special experiments urine and blood serum were also employed.

Tables II and III and particularly IV show wide variations in resistance of the various organisms to the tellurium diketones. The concentration of phenol necessary to destroy a given organism generally varied but little; often the range was within $\pm 5\,\%$ of the mean bactericidal concentration. The resistance of the organisms to the tellurium compounds, on the contrary, showed wide variations, an extreme case of this being that of *B. typhosus* ii (Table II) with which the bactericidal concentration of tellurium dipropionylmethane varied from 1 in 5,000,000 to 1 in 40,000,000. In investigating the bactericidal action of these compounds many tests must therefore be made and the substances examined as far as possible at the same time and under the same conditions. Accordingly the tabulated data are the mean results which have been obtained from several experiments.

Bactericidal Action on *B. coli communis* in Broth.

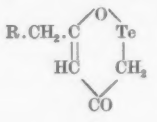
For the purposes of ascertaining the most efficient germicides, and for elucidating general principles of structure, two strains (i and ii) of *B. coli communis* were employed as the test organism. Whenever a strict comparison between the bactericidal powers of two or more substances was required, the tests were carried out on the same day, with the same broth culture of the organism. Such comparisons were found to hold consistently whenever both substances acted on the same culture, even though the actual values obtained varied widely from one experiment to another. In all some sixteen compounds were tested with *B. coli communis* in broth and the results are given in Table I.

Table I. *Bactericidal action on* B. coli communis *in broth.*

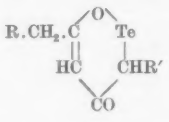
	Tellurium β-diketone	Mean bactericidal concentration	Equimolecular phenol coefficient
1.	Tellurium acetylacetone	1 in 500,000	2,300
2.	,, 3-methylacetylacetone	1 in 900,000	4,300
3.	,, 3-ethylacetylacetone	1 in 2,500,000	12,700
4.	,, 3-*n*-butylacetylacetone	1 in 2,800,000	14,500
5.	,, 3-methylpropionylacetone	1 in 5,000,000	25,400
6.	,, 3-ethylpropionylacetone	1 in 2,000,000	10,700
7.	,, dipropionylethylmethane	1 in 2,800,000	14,500
8.	,, propionylacetone	1 in 3,000,000	14,400
9.	,, *n*-butyrylacetone	1 in 3,000,000	15,200
10.	,, *n*-valerylacetone	1 in 1,200,000	6,400
11.	,, *n*-hexoylacetone	1 in 700,000	3,900
12.	,, *n*-heptoylacetone	1 in 500,000	2,900
13.	,, dipropionylmethane	1 in 9,000,000	45,800
14.	,, propionyl-*n*-butyrylmethane	1 in 3,000,000	16,000
15.	,, di-*n*-butyrylmethane	1 in 5,000,000	27,000
16.	,, 3 : 3-diethylacetylacetone	1 in 900,000	5,100

From an examination of Table I a certain amount of data as to the effect of chemical structure on the bactericidal action of tellurium compounds on *B. coli communis* may be derived.

Homologous series. (*a*) Tellurium compounds derived from the diketones $R.CH_2.CO.CH_2.CO.CH_3$ furnish the best example of homologous series, rising from tellurium acetylacetone to tellurium *n*-heptoylacetone (Nos. 1, 8, 9, 10, 11, 12). These substances have the general formula I, where R = H



I.



II.

in the parent compound and $R = C_5H_{11}$ in tellurium *n*-heptoylacetone. The equimolecular germicidal power rises rapidly from initial member (1) to the next (8), there is a slight increase in power on the part of (9), and then the germicidal power of the next three homologues diminishes steadily, (12) having nearly the same activity as (1). In this series the bactericidal power reaches a limit when $R = C_2H_5$, in tellurium *n*-butyrylacetone.

(*b*) Tellurium compounds derived from the diketones

$$R.CH_2.CO.CH_2.CO.CH_2R'$$

have the general formula II:

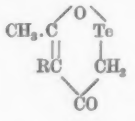
(1) When R = R′, the initial member (1) has a weak bactericidal power, but an enormous increase in power follows the substitution of two methyl groups to give tellurium dipropionylmethane (13). This substance has easily the highest germicidal power of the tellurium compounds yet studied. The conversion of R and R′ into ethyl radicles in (15) results in a considerable diminution in efficacy.

(2) When R is greater than R′ by one methylene group, as exemplified by (8) and (14), the higher homologue is slightly more powerful than the lower.

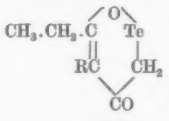
(*c*) Tellurium compounds derived from the diketones

$$CH_3.CO.CHR.CO.CH_3$$

have the general formula III and are represented by compounds (1), (2), (3) and (4), in which R rises from H to C_4H_9. There is a steady rise in germicidal



III.



IV.

power as the series is ascended up to tellurium 3-ethylacetylacetone but the 3-*n*-butyl compound has only bactericidal value of the same order as the former substance.

(*d*) Tellurium compounds derived from diketones

$$C_2H_5.CO.CHR.CO.CH_3,$$

having the formula IV, show a decrease in germicidal power as the homologous series rises from (5) to (6)—R = CH_3 and C_2H_5 respectively—which is a marked contrast to series (*c*) and evidently the result of the change from acetylacetone to propionylacetone as the starting-point.

Function of Alkyl Groups.

A consideration of the diverse effects on bactericidal power obtained by using members of various homologous series leads to the following conclusion. The conversion of tellurium acetylacetone into tellurium propionylacetone, resulting in the formation of an ethyl group, corresponds with a considerable rise in bactericidal power. The subsequent introduction of one methyl group in any of three possible positions causes a further rise in germicidal efficacy. Any further substitution of alkyl then causes a diminution in bactericidal power.

This generalisation is shown clearly by the following comparisons:

		Equimolecular phenol coefficient
1.	Tellurium propionylacetone	14,400
	„ 3-methylpropionylacetone	25,400
	„ 3-ethylpropionylacetone	10,700
2.	„ propionylacetone	14,400
	„ *n*-butyrylacetone	15,200
	„ *n*-valerylacetone	6,400
3.	„ propionylacetone	14,400
	„ dipropionylmethane	45,800
	„ propionyl-*n*-butyrylmethane	16,000

The accumulation of alkyl groups thus causes a rise in bactericidal activity, followed by a diminution, the maximum effect being caused by the formation of the ethyl group enhanced by the further addition of one methyl group. The effect of accumulation of alkyl groups upon physiological action is of interest in this connection. Generally speaking, the intensity of action increases steadily as the homologous series is ascended, but examples are to be found in which a limit is reached, in much the same way as with the bactericidal action of the tellurium diketones. Similar constitutive effects have been observed in the veronal group of drugs where the maximum effect is reached at the propyl derivative (proponal).

Isomerism.

The change in bactericidal power of a tellurium compound induced by introducing an alkyl group varies considerably according to the orientation of this substituent. These tellurium derivatives fall into three main groups, those in which substitution in the parent acetylacetone is

(*a*) symmetric, as in formula $R.CH_2.CO.CH_2.CO.CH_2R$;

(*b*) dissymmetric, as in formula $R.CH_2.CO.CH_2.CO.CH_2R'$;

(*c*) central, as in formula $R.CH_2.CO.CHR''.CO.CH_2.R'$.

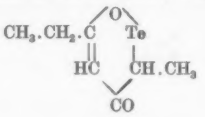
The following are representative cases of isomerism, the figures in brackets being the equimolecular phenol coefficients of the substances.

	Symmetric	Dissymmetric	Central
1.	—	Tellurium propionylacetone (14,400)	Tellurium 3-methylacetylacetone (4,300)
2.	Tellurium dipropionylmethane (45,800)	Tellurium *n*-butyrylacetone (15,200)	Tellurium 3-ethylacetylacetone (12,700) Tellurium 3-methylpropionylacetone (25,400)
3.	—	Tellurium *n*-valerylacetone (6,400) Tellurium propionyl-*n*-butyrylmethane (16,000)	Tellurium 3-ethylpropionylacetone (10,700) —
4.	Tellurium di-*n*-butyrylmethane (27,000)	Tellurium *n*-hexoylacetone (3,900)	Tellurium dipropionylethylmethane (14,500) Tellurium 3-*n*-butylacetylacetone (14,500)

In each case it is evident that symmetric substitution is more effective than any other type and that in general those tellurium compounds of which the parent diketones show the nearest approach to terminal symmetry are the most potent germicides. For instance, tellurium propionyl-*n*-butyrylmethane is more powerful than tellurium *n*-valerylacetone. The introduction of a single alkyl group into the terminal position is generally associated with greater germicidal power than the introduction of the same group into the central 3 position, except in the case of more dissymmetric derivatives.

Substitution by two separate methyl groups is more valuable than substitution by one ethyl group, since this leads to a nearer approach to symmetric terminal substitution (see example 2).

Consideration of the effects of homologous series and isomerism on the germicidal power of tellurium diketone derivatives shows that there are two main conditions necessary for high germicidal power. These are (1) the formation of an ethyl group followed by the further introduction of a methyl group in the para-position; (2) symmetric terminal substitution of the parent diketone. Tellurium dipropionylmethane (formula V), which is derived from the diketone $CH_3.CH_2.CO.CH_2.CO.CH_2.CH_3$, alone fulfils both these conditions and this is probably the explanation of its outstanding bactericidal power.



V.

Bactericidal Action with Organisms other than *B. coli communis*.

Broth inhibitory tests were carried out on various tellurium compounds with *B. typhosus* and with *Staphylococcus pyogenes aureus*. Two strains of *B. typhosus* were employed, strain (i) being a somewhat old culture, and (ii) a more recently isolated strain. The germicidal values obtained are given in Table II.

Table II. *Bactericidal action of tellurium β-diketones on* B. typhosus i.

Tellurium β-diketone	Mean bactericidal concentration	Equimolecular phenol coefficient
Tellurium acetylacetone	1 in 900,000	3,300*
„ 3-methylacetylacetone	1 in 1,500,000	5,900
„ 3-ethylacetylacetone	1 in 2,200,000	9,100
„ 3-methylpropionylacetone	1 in 5,000,000	21,000
„ propionylacetone	1 in 2,300,000	9,000
„ *n*-butyrylacetone	1 in 2,400,000	10,000
„ dipropionylmethane	1 in 7,500,000	31,000
„ propionyl-*n*-butyrylmethane	1 in 3,000,000	13,000
„ di-*n*-butyrylmethane	1 in 3,000,000	13,800
„ 3 : 3-diethylacetylacetone	>1 in 500,000	< 2,200

Bactericidal action of tellurium β-diketones on B. typhosus ii.

Tellurium 3-methylpropionylacetone	1 in 8,000,000	33,000*
„ 3-ethylpropionylacetone	1 in 2,500,000	11,000
„ dipropionylmethane	1 in 20,000,000	83,000

* Mean bactericidal concentration for phenol = 1 in 650.

It will be seen from Table II that *B. typhosus* i was less sensitive to change in chemical structure than *B. coli communis*, the smaller difference in activity between tellurium acetylacetone and tellurium dipropionylmethane being clearly noticeable. Nevertheless, the general rules with regard to substitution and structure hold in this case as with *B. coli communis*.

Both strains were equally resistant to phenol, but *B. typhosus* ii was much more susceptible to the tellurium compounds than the former strain, although the same chemical relationships apply as before.

The experiments with *Staphylococcus pyogenes aureus* (Table III) show that the two strains, i and ii, have entirely different resistances to the action of the tellurium compounds; notwithstanding this, however, the comparative relationships already observed between the germicidal powers of these compounds are still maintained.

Table III. *Bactericidal action of tellurium β-diketones on* Staphylococcus pyogenes aureus i.

Tellurium β-diketone	Mean bactericidal concentration	Equimolecular phenol coefficient
Tellurium acetylacetone	1 in 18,500	98*
„ 3-methylacetylacetone	1 in 42,000	240
„ 3-ethylacetylacetone	1 in 112,000	680
„ propionylacetone	1 in 400,000	2,300
„ *n*-butyrylacetone	1 in 450,000	2,700
„ dipropionylmethane	1 in 900,000	5,400
„ propionyl-*n*-butyrylmethane	1 in 620,000	3,900

* Mean bactericidal concentration for phenol = 1 in 450.

Bactericidal action of tellurium β-diketones on Staphylococcus pyogenes aureus ii.

Tellurium *n*-butyrylacetone	1 in 4,000,000	20,000*
„ dipropionylmethane	1 in 6,000,000	30,000
„ dipropionylethylmethane	1 in 5,000,000	25,000

* Mean bactericidal concentration for phenol = 1 in 535.

The explanation of the above remarkable divergence depends on the fact that the resistances of *Staphylococcus* and *Streptococcus* vary considerably at different periods of their life-history. In Table IV the results with *Staphylococcus pyogenes aureus* (i a) and *Streptococcus haemolyticus* were obtained when the organisms had been freshly cultivated on artificial media from the animal body. Under these conditions the cocci are so highly resistant that the tellurium compounds are seen to act selectively on organisms of the coliform group, thus resembling quinones as previously mentioned [Morgan, Cooper and Burtt, 1923]. On further cultivation in artificial media, however, the resistance of these organisms to tellurium derivatives diminished five to ten times, but in their resistance to phenol there was practically no change. Organisms of the coliform group did not exhibit this remarkable change in susceptibility. It is interesting also to note that the final resistance to tellurium dipropionylmethane of the *Staphylococcus* (i b) after cultivation in artificial media was of the same order as that of the other strains (ii) and (iii), which had also been grown for some time in laboratory media.

Bactericidal Action of Tellurium dipropionylmethane on a Variety of Organisms.

Tellurium dipropionylmethane, having by far the greatest germicidal power, was tested by the inhibitory method in broth with a considerable number of species of bacteria including bacilli (coliform and otherwise), cocci and the acid-fast group. The results are arranged in Table IV.

B. phlei was taken to represent the acid-fast group including tubercle and leprosy. This type of organism caused a difficulty in technique, since the culture grows in lumps or flakes, and not as a homogeneous suspension.

To this tellurium compound *B. pyocyaneus* showed a very marked resistance which may be associated with the extremely rapid growth of the organism.

Table IV. *Bactericidal action of tellurium dipropionylmethane in broth.*

Organism	Mean bactericidal concentration	Equimolecular phenol coefficient
B. coli communis i	1 in 9,000,000	46,000
„ ii	1 in 10,000,000	51,000
B. typhosus i	1 in 7,500,000	31,000
„ ii	1 in 20,000,000	83,000
B. paratyphosus A	1 in 17,000,000	57,000
„ B	1 in 4,000,000	22,000
B. pyocyaneus	1 in 250,000	800
B. phlei	1 in 750,000	4,500
Staphylococcus pyogenes aureus i a	1 in 900,000	5,400
„ „ i b	1 in 10,000,000	46,000
„ „ ii	1 in 8,000,000	46,000
„ „ iii	1 in 6,000,000	30,000
Streptococcus haemolyticus a	1 in 540,000	2,900
„ „ b	1 in 2,800,000	13,500

The remarkable bactericidal power of tellurium dipropionylmethane indicated in Table IV is also well exhibited by a comparison with that of other

well known disinfectants, as shown by the inhibitory test, using *B. coli communis*, the numbers indicating germicidal concentration: phenol 1 in 530, mercuric chloride 1 in 2,000,000, benzoquinone 1 in 20,000, tellurium dipropionylmethane 1 in 8,000,000.

Bactericidal Action of the Tellurium β-Diketones in Media other than Broth.

In order to test the applicability of the substances to internal disinfection, inhibitory tests, for the most part with tellurium dipropionylmethane, were carried out in blood serum and in urine.

Test in blood serum. The serum employed was ox blood serum, heated at 56° to destroy the complement and to produce sterility. The inhibitory test was carried out in the usual way and the serum tubes subcultured into broth after two days' incubation. The test organism was grown in serum from nutrient agar for 24 hours. It was found that the compounds lost their efficacy in serum to a remarkable degree. Thus tellurium dipropionylmethane failed to kill either strain of *B. typhosus* in a concentration of 1 in 50,000.

It was hoped that tellurium 3 : 3-diethylacetylacetone would give better results, since it cannot function as a weak acid, thus differing from the other derivatives. Nevertheless, this substance failed to kill *B. typhosus* in a concentration of 1 in 50,000. Tellurium 3-methylpropionylacetone also failed to show bactericidal action in a similar concentration.

This collapse of germicidal power in serum employed as medium could be the result of either or all of three factors: (i) change in cultural characteristics of organism, due to growth in serum; (ii) change in hydrogen ion concentration, serum being on the alkaline side, whereas the broth used was acidic; (iii) complex reaction or decomposition of the tellurium compound with serum proteins.

(i) On growing the two strains of *B. typhosus* in serum, and using this culture for a broth inhibitory test, it was found that the resistance of the organisms was not noticeably different from the normal.

(ii) The use of broth of p_H equal to that of serum in the inhibitory test caused no very definite change in bactericidal power.

(iii) A broth inhibitory test was carried out, using a broth culture of *B. typhosus*. In one case tellurium dipropionylmethane solutions were made up in water as usual, and in another case in serum (1 cc. of 0·01 % aqueous solution was added to 9 cc. of serum to make a 0·001 % solution in serum), the serum solution being left for one hour before use. The control experiment gave a normal result (sterilisation in 1 in 10,000,000), while that in which a serum solution of the tellurium derivative was added to the broth showed that the substance had lost its bactericidal action in concentrations down as far as 1 in 500,000. A control experiment, in which the tellurium dipropionylmethane was dissolved in broth instead of in water, showed no divergence from that in which water was used.

It is thus seen that in some way the serum had succeeded in rendering the tellurium dipropionylmethane non-bactericidal, and it is evidently factor (iii), the protein content of the serum, which is responsible for the diminution in activity of these compounds in serum.

We have nevertheless shown that tellurium dipropionylmethane in serum is capable of inhibiting growth to a considerable degree, even in concentrations much less than those in which it fails to be germicidal. The rate of increase in numbers of *B. typhosus* in ordinary serum and in serum containing one part of tellurium dipropionylmethane per million was compared. The two tubes were each inoculated with one drop of a serum culture of *B. typhosus* and incubated. The numbers of organisms present in each tube were counted by plating on agar at the beginning and after three and nine hours. The results were as follows:

Pure serum	Serum + Tellurium dipropionylmethane
Beginning of experiment:	
30,000,000 organisms per cc.	26,000,000 organisms per cc.
After three hours at 37°:	
70,000,000 organisms per cc.	25,000,000 ,,
After nine hours at 37°:	
90,000,000 organisms per cc.	31,000,000 ,,

The experiment shows that in the presence of one part of tellurium dipropionylmethane per million growth of the organism is very slow.

Tests in urine. The urine was sterilised before use by steaming and the test organisms used were grown in urine for 24 hours instead of broth. Tellurium dipropionylmethane was examined with various organisms and the following results were obtained:

Organism	Mean bactericidal concentration	Organism	Mean bactericidal concentration
B. coli communis ii	1 in 5,500,000	*B. paratyphosus* A	1 in 9,000,000
B. typhosus i	1 in 3,300,000	,, B	1 in 6,000,000
,, ii	1 in 4,000,000	*S. pyogenes aureus* ii	1 in 2,900,000

The results show that whilst there is a perceptible diminution of activity in urine as compared with broth, on the whole the results are quite of the same order (see Table IV), and tellurium dipropionylmethane has a very pronounced action in urine.

Physiological Action of Tellurium β-Diketones.

Experiments to determine the toxicity to animals of certain of these substances were next carried out with a view of ascertaining whether there was any likelihood of them being suitable for administration to man as internal disinfectants.

Method. The animals used were mice (weight approximately 20 g.). The tellurium compounds in aqueous solution were injected subcutaneously into the back leg, with the usual precautions against sepsis. The solutions were of such strength that the required dose was contained in 0·2 to 0·25 cc. The following doses were found to be fatal:

Tellurium β-diketone	Actual dose (g.)	Dose per kilogram body weight (g.)
Tellurium dipropionylmethane	0·00010	0·005
,, 3-methylpropionylacetone	0·0003	0·014
,, 3-ethylacetylacetone	0·000375	0·013

These substances are therefore extremely poisonous and it is evident that tellurium dipropionylmethane is the most toxic of the three isomerides, while central substitution of the diketone gives less toxic compounds. Bactericidal power and toxicity are thus similarly influenced by chemical structure.

The general symptoms and behaviour of mice suffering from poisoning by these substances were similar in each experiment. With the dose well below the toxic value no symptoms were observable. As the dose was increased the mouse usually showed signs of slight *malaise*, and a small quantity of blood would be passed in the urine a few hours after the injection. Normal recovery took place in about two days. When a lethal dose had been injected the mouse speedily showed signs of *malaise*, hematuria followed, and the animal soon died.

The post-mortem examination of mice which had succumbed showed similar conditions in every case. The lungs and heart did not have an unusual appearance. The liver was sometimes enlarged slightly; the kidneys were also slightly swollen and abnormally dark in colour and the spleen was almost invariably enlarged.

In the case of one experiment, the animal was dissected within 30 minutes after death, its organs were preserved in formalin and submitted to a medical colleague for histological examination. Microscopic examination of the tissue showed that the animal had died from some generalised toxic condition which affected in particular the spleen, liver and kidney. Quite the most characteristic symptom of poisoning by a tellurium diketonic derivative is hematuria, which in extreme cases is probably the primary cause of death.

Further experiments showed that these substances are cumulative in large doses but not in small doses. Thus, a dose of 0·10 mg. of tellurium 3-methylpropionylacetone caused decided but not fatal symptoms, while in another experiment the same quantity, divided into three doses, given at two days' intervals, caused no apparent symptoms. The same compound, however, caused slight symptoms following a dose of 0·125 mg., and a second injection of the same quantity two days later resulted in marked symptoms of severe poisoning.

Possibility of the Therapeutic Application of Tellurium Derivatives.

The foregoing experiments with tellurium diketones in serum show that these substances would be of little value in the blood stream unless their action as growth inhibitors only was desired.

On the other hand, the tellurium compounds retain their efficacy in urine and it would appear possible that they might be of use in treatment of infections of the urinary organs, and clinical tests are in progress. In the case

of mice, the lethal dose of tellurium dipropionylmethane is 0·005 g. per kilogram body weight, that is, a concentration of 1 in 200,000 if we suppose the substance to be distributed evenly throughout the body. Experiments have shown that a concentration of 1 in 2,000,000 in urine is more than sufficient to kill any of the organisms examined.

Thus the ratio between lethal concentration and bactericidal concentration is over ten in the case of tellurium dipropionylmethane. Lethal dose and bactericidal concentration of tellurium 3-methylpropionylacetone (the substance of the next greatest bactericidal power) are both rather higher than those of the former substance, but the ratio between lethal and bactericidal concentrations seems to be about the same.

The two compounds which appear to be most suitable for therapeutic application are tellurium dipropionylmethane and tellurium 3-methylpropionylacetone; the necessary concentration of the former would be less, but the latter, a more easily prepared compound, is more readily soluble in water.

Bactericidal Action of Telluric Acid.

For purposes of comparison with the researches on tellurium diketones, the bactericidal power of telluric acid by the inhibitory test in broth and blood serum has been determined, with the following results:

Broth	Bactericidal concentrations	Blood serum	Bactericidal concentrations
B. coli communis	1 in 3,000	*B. coli communis*	1 in 900
B. typhosus	1 in 5,000	*B. typhosus*	1 in 900
S. pyogenes aureus	1 in 30,000 1 in 50,000		

The results show that telluric acid is much less active than the diketone compound but has a remarkable selective germicidal action on *S. pyogenes aureus* which is quite without parallel in the study of the above substances. The bactericidal action of telluric acid is decidedly less in serum than in broth, but the germicidal power in serum does not show the complete collapse characteristic of the diketone derivatives. Joachimoglu [1922] found telluric acid to be germicidal to coliform organisms in concentrations of 1 in 40,000, but in our experiments we have found it to be less active.

Summary.

1. The tellurium derivatives of the aliphatic β-diketones are extremely powerful germicides to many different types of bacteria.

2. Two conditions of chemical structure are mainly contributory to high bactericidal power, namely (*a*) symmetry of the parent β-diketone, (*b*) presence of methyl and ethyl groups in para-positions within the heterocyclic ring. These conditions are simultaneously fulfilled only in the case of tellurium dipropionylmethane, which is therefore the most active of the series, being sometimes bactericidal in the case of *B. typhosus* in as low a concentration as 1 in 40,000,000.

3. The bactericidal power of these compounds increases considerably as the homologous series is ascended, until with the formation of an ethyl group and subsequent substitution of one methyl group, a limit is reached, and with further substitution of alkyl groups there is a diminution in efficacy.

4. Position isomerism plays an important part in determining germicidal power, the relative positions of the alkyl groups in the molecule considerably affecting the activity of these compounds.

5. With such powerful germicides as those under consideration, small cultural changes in an organism or small differences between various strains of the same organism may profoundly affect the results.

6. The tellurium compounds lose their strong bactericidal action in the presence of serum, but retain their inhibitory power.

Their *germicidal* action, however, is retained in urine.

7. The compounds are very toxic to mice, attacking the kidneys in particular, causing hematuria. Differences of toxicity appear to correspond with differences in bactericidal power.

8. It would appear possible that the tellurium diketones, and particularly tellurium dipropionylmethane and tellurium 3-methylpropionylacetone, may find application in medicine as urinary antiseptics.

The authors desire to express their thanks to the Advisory Council of the Department of Scientific and Industrial Research for grants which have helped to defray the expense of this investigation.

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XXX. THE NATURE OF THE RED BLOOD CORPUSCLE.

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(*Received January 1st, 1924.*)

This investigation began as an attempt to elucidate the process of haemolysis, but it soon became clear that it was necessary first to understand the organisation of the corpuscle. The subject concerns biologists, not only for its own sake, but also for the light which it throws on the nature of animal cells in general; physicists, too, should be interested by the hypothesis that the red corpuscle is a non-spherical liquid droplet.

Historical.

Since the early days of microscopy, the question of the solid or liquid nature of the red blood corpuscle has excited interest, and opinion has fluctuated between the two hypotheses.

Leeuwenhoek [1798] states: "The blood is composed of exceeding small particles, named globules, swimming in a liquor called the serum," and "Particles...larger in fishes, they being of a flat and oval shape; whereas on the contrary, so far as I could judge from my eye, they in this animal (the bat) were spherical."

For a long time mammalian corpuscles were held to be spherical globules. Hewson [1777] was the first to recognise that human red corpuscles are in reality "flat bodies." This proves, he concluded, that they are not fluid, "because every fluid swimming in another which is in larger quantity, if it be not soluble in that other fluid, becomes globular." Yet he decided to call them "flat vesicles."

The conception of fluid vesicles was maintained by De Blainville [1829]: "The Globules are formed of a little bladder filled with fluid...in which is found the colouring matter; and each of these globules encloses others smaller, though of similar structure." He appears to have had the erroneous idea that they contain nuclei.

Schwann [1839] made the same mistake in believing that mammalian red blood corpuscles contain a nucleus. He says, quaintly: "If they were but a very little smaller they would escape observation altogether, and the blood corpuscles would then appear to be cells without nuclei." His anxiety to reduce all animal tissue to the type of nucleated cells caused him to stretch the evidence to support his theory. His idea that animal cells are typically

vesicles (the idea arising from comparison with plant cells) led him to believe that red blood corpuscles are vesicles. This belief was strengthened by the observation, first made by Hewson, that the nucleated corpuscles of the toad swell in water and become round, and the nucleus can be seen to roll about "like a pea in a bladder." In another matter he anticipated a very recent discovery. Pointing out that the cell contents differ from the surrounding medium, he says: "It might perhaps be conjectured from this peculiarity of the metabolic phenomena in cells, that a particular position of the axes of the atoms (? meaning molecules) composing the cell-membrane is essential for the production of these appearances." This exactly accords with the most modern views of molecular orientation at liquid surfaces [Clayton, 1923].

In recent years, Sharpey-Schäfer [1912] has strongly maintained the hypothesis of the fluid nature of the corpuscles, for the following reasons. (1) In hypotonic solutions they assume a globular form; whereas a solid body imbibing water would, in swelling, retain its former shape. (2) As the corpuscles circulate in the capillaries, slight variations of pressure cause distortion, which would be impossible if they were solid. (3) In the corpuscles of ovipara the nucleus readily becomes displaced.

Koeppe [1903] supports the same hypothesis, but not unreservedly: "The representation of a red blood corpuscle as a bladder filled with fluid contents is quite reasonable; it agrees very well with the observed appearances, but may not correspond to reality."

Most recent writers, following the lead of Rollett [1900] and Hamburger [1898] favour the hypothesis of a stroma containing endosoma in its pores. The great stumbling-block which has prevented acceptance of the fluid hypothesis is that which Hewson recognised, and which is re-stated by Rollett as follows: "How can one imagine that a bladder with a flexible membrane and floating in a liquid, should assume a discoid shape?" An answer to this question was given by Norris, and the point will be elaborated later. Rollett describes appearances in corpuscles treated with water or with solution of ammonium chloride, which he interprets as indicating the presence of a stroma; in numerous microscopical observations of normal blood, I have never seen anything which conveyed the same idea to my mind.

The work of Norris [1882] is referred to last, because he appears to have approached most nearly to the truth. His conclusions have not received the attention they deserve, probably because they were associated with some erroneous views on haematogenesis. The following quotations will make his position clear:

"The extensibility and retractility of blood corpuscles is that of liquids, and the elasticity of such a substance as caoutchouc is by comparison rigidity itself. If we could separate this substance into minute masses of the size of red corpuscles, they would, when submerged in liquid, appear as absolutely rigid bodies. The small motor influences, such as currents, impacts, etc., which are in operation in a specimen of blood, would not affect their form in the least. Even oil globules of the same size as the blood corpuscles are inextensible

under such delicate influences, and if the so-called stroma was no more limpid than oil the corpuscles could not possibly display any elasticity."

"The remarkable properties displayed by myelin (lipoids) at once relieve us from the necessity of considering that one liquid or solution submerged in another must inevitably take on the globular or spheroidal state. The fact is, this substance appears to represent the extending or spreading-out tendency, as opposed to the gathering-up or sphere-forming property. The biconcave and the annular forms seem to be related to a kind of balancing of these two properties."

The Corpuscle a Liquid Droplet.

Our knowledge of vital processes leads us to expect the liquid state, which would allow freer diffusion in the interior of the corpuscles. Pascucci's observation [1905] that haemoglobin crystals may separate freely inside a corpuscle, indicates the absence of internal septa. Reference has already been made to the reasons adduced by Sharpey-Schäfer. Very strong evidence is furnished by the following experiment. Although the observation is so simple, it has apparently never before been made; at least it has not to my knowledge been placed on record. When we consider the number of investigations, such as Wassermann tests, in which washed blood corpuscles are used, it is wonderful that the alteration in shape, when a change is made in the medium of suspension, should have escaped notice[1].

Exp. 1. A small quantity of blood is obtained from a puncture in the ear or finger, and it is allowed to stand until the clot has separated from the serum. The clot is then picked out, placed in a centrifuge tube and teased until most of the corpuscles have escaped from the fibrinous mesh, which is then removed. If some corpuscles and serum are mixed on a slide in the proportion of 1 in 50, covered, and examined with a 1/12th inch oil-immersion objective, a clear view is obtained of the red corpuscles in the form of biconcave discs with rounded edges. The corpuscles are washed in three changes of 0·85 % solution of sodium chloride, in order to free them from serum. The tube containing the serum is also spun in order to throw down any corpuscles which may remain in it. We have now some serum free from corpuscles, and some corpuscles suspended in normal saline solution. A microscopic preparation of the latter (1 in 50) is made and examined as before. It is seen that the corpuscles are now all in the form of spherical droplets, with a diameter about a quarter less than that of the corpuscles in their usual form. There is no "double contour." The stage of the microscope may be tilted and the cover glass touched with the point of a needle, so as to cause some commotion in the film; but however much the corpuscles move about, no phase is seen other than the single phase presented by a sphere. Any possible doubt will be dispelled by seeing the spherical and discoid forms side by side, as will be described later.

[1] Norris [1882] was familiar with the spherical shape in saline solutions, and gives photomicrographs of the same, but he did not know the reverse change.

Next, some of the washed corpuscles are mixed with serum in the proportion of 1 in 50 and a microscopic preparation made. It is seen that the corpuscles have nearly all resumed their former discoid shape. The familiar double contour and all the phases of the normal corpuscles are seen. A certain number fail to regain their normal shape, but remain as spherical droplets; in a preparation carried through without delay, not more than 1 or 2 % fail, but the proportion is greater when they have remained for some hours in the saline solution. The change of shape takes place almost instantaneously, and occurs equally well at 15° and at 37°.

It seems impossible to account for these appearances except on the hypothesis that the red corpuscle is a liquid droplet. If only the first part of the observations were considered, it might be supposed that the corpuscles change from solid to liquid by reason of some damage sustained while being washed. But the reverse change would remain unexplained: if the liquid droplet were to re-solidify on re-suspension in serum there would be no reason why it should change its shape.

A considerable number of variations of this experiment have been tried. If the corpuscles are left for a few hours in isotonic saline solution, most of them become crenated; after standing 24 hours, some of them are again discoid, the probable explanation being that a rearrangement of constituents causes a diminution of surface tension. Stronger saline solutions favour the discoid form, and the most effective salts were found to be ammonium oxalate and ammonium chloride. 1 % of either of these, added to a 0·85 % solution of sodium chloride, gives a medium in which corpuscles are nearly all discoid. It may be pointed out that these salts of ammonium readily pass through the envelope of the corpuscle, and are therefore available for reducing surface tension both from without and from within.

The serum of other animals has the same effect as homologous serum, but egg-albumin and a solution of haemoglobin proved ineffective. Dilutions of serum were tried; it was found that serum, diluted with normal saline solution in the proportion of 1 in 8, causes many corpuscles to return to their normal shape, but a dilution of 1 in 16 has little effect. In the first case spheres, discs and crenated forms can all be seen in the same preparation. There are scarcely any forms intermediate between spheres and the normal biconcave discs, just a very few oval biconvex discs: it seems as if the balance nearly always inclines one way or the other, and is very rarely found poised in a neutral position.

The Contents of the Corpuscle.

The corpuscle contains about 30 % of solids, nearly all haemoglobin: the lipoids are no more than 1 % of the total solids. From general principles, we should expect a hydrophile colloidal system, for most constituents of animal tissues are included in this class: the dispersed phase would consist of haemoglobin with water and probably salts; the continuous phase would consist of

water and salts. The following experiment, in which Hedin's [1895] haematocrit method was employed, gave results which favour this view.

Exp. 2. Some sheep's blood was taken and clotting prevented by the addition of half its volume of a 2 % solution of sodium citrate. The corpuscles were freed from serum by being washed in three changes of normal saline solution. Nearly all the supernatant fluid was removed, only sufficient being left to allow the suspension to be manipulated with a Wright's capillary pipette. A series of ten solutions of sodium chloride of various strengths from 0·6 % to 4·0 % was prepared, and equal volumes of each solution were measured into a series of small glass test-tubes. The same volume of corpuscular suspension was added to each tube, and thoroughly mixed with the solution of sodium chloride. By means of a very fine capillary pipette, these mixtures were transferred to a series of haematocrit tubes, each being filled to a height of 10 cm. and labelled. As haematocrit tubes, pieces of quill-tubing, 11 cm. long with 1 mm. bore, sealed at one end, were used. The whole were then spun for 3 hours in a water-centrifuge: at the end of that time, the height of the deposit in each tube was measured.

It is necessary to calculate the percentage of sodium chloride in the solutions at the end of the experiment. In each tube were placed at the beginning 5·0 cm. of sodium chloride solution of various strengths and 5·0 cm. of suspension of corpuscles. From the deposit in tube 4, it is seen that the amount of corpuscles was 3·0 cm., and therefore 2·0 cm. of 0·85 % solution of sodium chloride was introduced along with them. The percentage of sodium chloride in any tube is therefore given by the formula

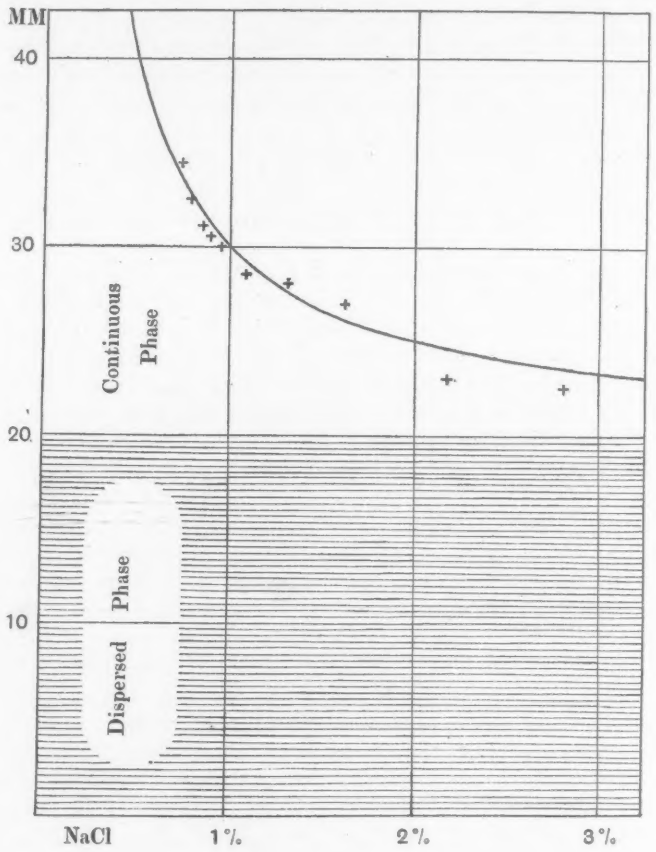
$$\frac{5 \times P + 2 \times 0{\cdot}85}{10 - D},$$

where P is the percentage of sodium chloride in the solution at first, and D is the height of the deposit in centimetres. This is not absolutely correct, since the solution in the interstices between the corpuscles is not taken into consideration; but the error is negligible. The results are given in the following table:

Tube	Sodium chloride solution used %	Calculated percentage at end	Height of sediment of corpuscles cm.
1	0·6	0·72	3·45
2	0·7	0·77	3·25
3	0·8	0·83	3·1
4	0·9	0·89	3·05
5	1·0	0·96	3·0
6	1·2	1·08	2·85
7	1·5	1·28	2·8
8	2·0	1·60	2·7
9	3·0	2·17	2·3
10	4·0	2·80	2·25

The results are represented graphically in the diagram, the ordinates representing volume of corpuscles and the abscissae concentration of solution: the observed results are marked by crosses. There is much evidence to show

that the envelope of the corpuscle is approximately "semipermeable" to most neutral salts such as sodium chloride. This being so, the volume of the contents concerned in osmotic equilibrium should vary inversely as the concentration of the surrounding fluid. It will be seen that the values do fulfil the requirements of the osmotic theory if we exclude a volume of 2·0 cm. (two-thirds of the normal bulk of the corpuscles) as taking no part in osmotic



equilibrium. The values calculated on this supposition are represented by the continuous curve and the observed values lie fairly close to it. Perfect agreement cannot be expected, for there are at least three sources of discrepancy: (1) as the corpuscles shrink, the increased concentration of saline contents may cause an alteration in the amount of water imbibed by the haemoglobin; (2) a gradual permeation of sodium chloride through the envelope, which is not

absolutely semipermeable; (3) alteration in surface tension and therefore in intracorpuscular pressure. We may conclude that the corpuscle contains a hydrophile colloidal system, the dispersed phase being haemoglobin (30 %) associated with 35 to 40 % of bound or imbibed water and probably with salts, the continuous phase being the remaining 30 to 35 % of water with dissolved salts.

The dispersed phase constitutes 65 to 70 % of the whole volume. Now, a large number of small equal spheres, packed as closely as possible without deformation, occupy 74 % of the total volume. Hence, if the dispersed phase is composed of equal spheres, they are not packed quite so closely as possible, and there is no serious impediment to free diffusion within the corpuscle. But the margin is small and it is easy to imagine that increased concentration of the body-fluids might impair the oxygen-carrying capacity of the corpuscles.

The Surface Film.

Pascucci [1905] showed that this is composed of lipoids and proteins. From physico-chemical principles, it is clear that substances which reduce surface energy will become concentrated at the surface, and this applies to substances from the interior of the corpuscle as well as those from the surrounding medium. It sometimes happens that such a concentration is so great as to lead to the formation of a solid surface film: we may now consider whether this is the case with the corpuscle.

When blood is laked by the addition of water, the envelopes of the corpuscles remain as "ghosts" and the appearance with high magnification suggests that these are collapsed bladders with extremely thin solid walls. The "ghosts" are seen both when blood is laked with cold water and when it is dropped into water at 37°: this indicates that the surface film is solid at the temperature of the body. "Ghosts" are also seen when blood-corpuscles, previously washed in normal saline solution at 37°, are laked in water at the same temperature or at room temperature. This shows that the surface film constituting a "ghost" is not removed by washing away the serum: if it is formed by solidification of constituents of the serum, re-solution does not readily occur.

The action of polyvalent ions throws light on the nature of the surface film. It is known that suspensoid colloids are agglutinated by polyvalent ions in extremely dilute solution, whereas emulsoid colloids are not nearly so sensitive; also, the ions causing agglutination carry an electric charge opposite in sign to that of the colloid particles.

Exp. 3. Sheep's blood was taken, and clotting prevented by the addition of half its volume of 2 % solution of sodium citrate. The corpuscles were freed from serum by being washed in six changes of 0·85 % solution of sodium chloride. A series of dilutions of the test substance was prepared, 0·85 % of sodium chloride being present in every one. About 0·2 cc. of each dilution was placed in a small tube and to each was added one platinum-loopful of the

washed corpuscles. The tubes were gently agitated until their contents were thoroughly mixed, they were then allowed to stand for 12 hours at room temperature. In the absence of agglutination the corpuscles settled as a sediment with a perfectly level surface, and none adhered to the sides of the tube above; agglutination was recognised by flocculi adhering to the sides of the tube.

By this method it was found that sheep's red corpuscles are agglutinated by

$CeCl_3$	...	...	1 in 1,000,000
$La(NO_3)_3$	...	...	1 in 1,250,000
$Th(NO_3)_4$	...	...	1 in 80,000
$FeCl_3$	...	...	1 in 300,000
$CaCl_2$	...	...	1 in 400
$K_4Fe(CN)_6$	...	...	1 in 100

These may be compared with the following results:

Human serum gave a precipitate with $CeCl_3$ 1 in 2000, but gave no precipitate with $K_4Fe(CN)_6$ 1 in 200.

Sheep's corpuscles laked by the addition of distilled water gave a slight precipitate with $CeCl_3$ 1 in 100,000. On microscopical examination, this precipitate was found to consist of leucocytes, with an amorphous substance in which "ghosts" could not be identified with certainty. On the surmise that lipoids might be concerned in the agglutination, a test was made with "Wassermann antigen" composed of the lipoids of heart muscle. It gave a precipitate with $CeCl_3$ 1 in 12,500, but none with $K_4Fe(CN)_6$ as strong as 1 in 125.

From these results it may be concluded that the red corpuscle has a solid surface film, which carries a negative electric charge when the medium of suspension is a solution of sodium chloride. The effective constituents of the film are probably the lipoids: it is true that these do not show agglutination or precipitation in such a high dilution as the corpuscles, but the phenomenon is more easily detected in the last, since a minute quantity of lipoid film has a relatively huge coloured content which renders any agglutination easily visible.

A rough calculation shows that if all the cholesterol and lecithin in the corpuscles were present as a continuous film on the surface, the film would be 2 to 3 $\mu\mu$ thick. As an atom of hydrogen is estimated to have dimensions of 0·25 $\mu\mu$ and a molecule of starch 5 $\mu\mu$, it is an interesting speculation that the lipoids may be present as a single layer of molecules.

Although the evidence points to the existence of a solid surface film, yet this may undergo changes, as will be suggested later in discussing crenation.

The *permeability* of the surface film has been the subject of a large amount of research, the results of which can only be summarised here [see Hedin, 1897; Gryns, 1896; Stewart, 1899, 1900; Rywosch, 1907]. Substances fall into four classes, according as they pass through the membrane freely, gradually, very slowly, or not at all. Freely permeating substances include water, urea, alcohols, ethers, esters and some ammonium salts: glycerol permeates gradually:

most neutral salts pass through very slowly indeed: the sugars are completely excluded. These differences depend not only on the size of the molecules, but also on their chemical nature.

The permeability is readily altered in many ways. The resisting power of the film is increased by hardening agents, such as formaldehyde and salts of silver, mercury and zinc. On the hypothesis of the corpuscle as a liquid vesicle, haemolysis is explained simply as the passage of the fluid contents through the damaged envelope, and it will be well to review the different kinds of haemolysis so as to find whether that explanation is adequate.

1. *Haemolysis in water* or in too dilute saline solutions is brought about by endosmosis of water until the distension is so great that the surface film becomes porous and allows the contents to leak out.

2. *Freezing and thawing.* Koeppe [1903] suggested that at some stage of the cycle, ice-crystals melt and a too dilute solution is formed in certain places: haemolysis then occurs as in (1). But cold may also alter the state of aggregation of particles in the surface film, and its permeability may be thereby increased.

3. *Heat.* An obvious explanation is that haemolysis is due to melting of the fatty film [Koeppe, 1903].

4. *Electric shocks* appear to act by mechanical injury to the envelope [Rollett, 1900]. Continuous currents cause haemolysis by the liberation of acid and alkali at the poles. Alternating currents cause a rise of temperature, which lakes the corpuscles.

5. *Fat solvents* remove some essential constituent of the film [Koeppe, 1903].

6. *Substances lowering surface tension*, such as saponin and bile-salt, probably displace the lipoids and so disintegrate the surface film.

7. *Inert substances* in the form of fine powders, such as barium sulphate and kaolin, adsorb some constituent of the film.

8. *Acids.* } These change the lipoids substances to fatty acids and soaps
9. *Alkalis.* } respectively.

10. *Strong saline solutions*, such as 10 % solution of sodium chloride, may damage the surface film by taking away water which is necessary for its integrity.

11. *Venoms and toxins.* }
12. *Natural haemolysins.* } In spite of the tremendous amount of research during the past quarter of a century,
13. *Specific haemolysins.* } it cannot be said that a completely satisfactory explanation is available, but the view that haemolysis is brought about by the disintegration of a surface film composed of a mosaic of lipoids and proteins is an attractive one. It may be that the specificity of these reactions is related to the special pattern of the mosaic as well as to the special nature of the proteins and lipoids. According to Taniguchi [1921], lipoids are an essential constituent of the "receptors" of sheep's corpuscles concerned in heterophile antiserum haemolysis, while those acted upon by isophile immune body are of protein nature.

14. *Spontaneous haemolysis.* One element appears to be a gradual permeation of salts into the interior of the corpuscles, until the surrounding solution becomes hypotonic.

It will be seen that explanations of all the varieties of haemolysis can be founded on the vesicular hypothesis.

The Characteristic Shape.

Calculation shows that a sphere having the same volume as a human red blood corpuscle would have a diameter of about 5μ. Its surface would be about 20 % less than that of the corpuscle: the discoid shape is therefore advantageous in presenting a larger surface for interchange between the plasma and the contents of the corpuscle. A more important consideration is that no point in the interior of the corpuscle is distant more than $0{\cdot}85\mu$ from the surface, whereas the centre of the spherical body would be $2{\cdot}5\mu$, or about three times as far, from the surface: the discoid form therefore favours rapid diffusion from the surface of the corpuscle to every part of the interior. There is a slight disadvantage in that the larger diameter needs a large calibre of capillaries.

The question arises how the corpuscle assumes its peculiar shape. It might be supposed that the vesicle has a solid envelope of a definite shape: it is difficult to reconcile this view with the observations described under Exp. 1 and with those to be described in connection with crenation: if this view were accepted, we should have no conception how the envelope took its definite shape in the first instance, and that would be as great a mystery as the one supposed to be elucidated.

I believe with Norris [1882] that "the Biconcave Shape is due to the operation of physical conditions and not to structural restraint." The simple geometrical figure suggests its production by a few simple factors. The envelope of the corpuscle is practically semipermeable; from which it follows, in accordance with the laws of osmosis and imbibition, that the volume of the corpuscle must have a definite value, which depends on the surface tension and on the tonicity of the surrounding medium. At the surface of the corpuscle, there is a solid film enclosed between two liquid films, those of the corpuscular contents and of the surrounding fluid. The solid film is elastic but its extensibility is limited. The tension of the two liquid films would reduce the corpuscle to a sphere, were there no opposing force. What is the nature of this opposing force? I suggest that it is a repulsion between the dispersed particles of the colloidal system in the interior of the corpuscle: it may be an electrostatic repulsion caused by the similar electric charges of the particles, or some chemical force may be responsible. There may be a similar state of affairs in the solid film itself (the "spreading-out tendency" of Norris). If the surface tension were very great compared with the repelling force, the shape would be spherical. When repulsion predominates, the result is the familiar biconcave disc with rounded edges.

It may be asked why the same phenomenon is not commonly seen in emulsions. In ordinary emulsions the droplets are homogeneous and forces of repulsion do not arise in their interior. As Norris pointed out, droplets composed of myelin may assume a biconcave or annular form. In the case of spheroidal cells, it is probable that their contents are composite, the different constituents neutralising the affinities of one another in such a way that forces of repulsion do not develop.

It is now possible to understand the change of shape seen in the first experiment. In serum there are many constituents which reduce surface energy and the surface tension is therefore very small: repulsion predominates, and the discoid shape results. With corpuscles washed free from serum and suspended in isotonic salt solution, the surface tension is much greater, the forces of repulsion are overcome, and the spherical form is produced. When the corpuscles are re-suspended in serum, the original conditions are present and the original form is resumed.

In the case of nucleated non-mammalian corpuscles, if we imagine an elongated nucleus floating in a corpuscle otherwise resembling that of a mammal, it is easy to see that the dispersive forces will cause the haemoglobin-system to occupy the periphery, and the nucleus will be forced to the centre. The oval form of camel's corpuscles is not yet explained.

Crenation.

This may affect the entire surface of the corpuscle or it may be limited to the margin of the disc. The processes may be very fine, resembling when seen under an oil-immersion lens the hairs on a gooseberry: from this there are all degrees of coarseness up to the stage where three or four processes take up the whole surface of the corpuscle. As a rule, the projections are conical, but sometimes they are rounded.

The conditions determining the state of crenation are elusive. Two preparations may be made as nearly alike as possible; one will show crenated corpuscles and the other will show none; in fact, in the same microscopic field, the corpuscles on one side may all be smooth and those on the other side all crenated. Corpuscles become crenated after standing for some time in normal saline solution or in serum: the phenomenon is often very striking when the medium of suspension is serum diluted with about eight times its volume of normal saline.

Crenated corpuscles can be brought back to the state of smooth spheres (in normal saline) by warmth, or by the action of dilute acid or alkali. The optimum temperature for producing the change is 45°: 40° is ineffective and 50° causes destructive changes. The strengths of hydrochloric acid and sodium hydroxide found best were 0·002 % and 0·01 % respectively, each being associated with 0·85 % of sodium chloride.

Crenation is usually explained as being a shrinking in hypertonic solutions.

This is evidently not the case, since it is best seen in isotonic solutions, and the wrinkling of corpuscles in hypertonic solutions is not the same thing.

The following explanation is suggested. The surface film of the corpuscle is under normal conditions uniform, at least so far as any microscopical examination can detect. With lapse of time and fall in temperature, there is liable to be a change in the state of aggregation of the micellae of the film: in some parts there may be denser masses and in other parts there may be rarefaction of the envelope; the change being analogous to agglutination or precipitation. The thin parts yield to the intracorpuscular pressure, and, according as the pattern of dense and thin portions is fine or coarse, so fine or coarse will be the crenations. When the temperature is raised, or in the presence of a trace of acid or alkali, the micellae spread out evenly and the original shape is resumed.

Summary.

1. The red corpuscle is a fluid droplet. The following evidence leads to this conclusion:

(*a*) Haemoglobin crystals separate freely in the interior of a corpuscle.

(*b*) The corpuscles assume a spherical form in hypotonic solutions.

(*c*) The nucleus of oviparous corpuscles is readily displaced.

(*d*) Slight forces produce deformation.

(*e*) In isotonic saline solutions a spherical form is assumed, and the reverse change to the discoid form takes place when the corpuscles are again placed in serum.

2. The contents form a hydrophile colloidal system. The change of volume in solutions of different concentration shows that the continuous phase (salts and water) constitutes one-third of the whole. The dispersed phase (haemoglobin, salts and water) forms the remaining two-thirds.

3. The surface film contains lipoids in a solid state; this is shown by the microscopic appearance of "ghosts," and by the agglutinating action of polyvalent kations in extremely dilute solutions. Its permeability by different substances is mentioned. The permeability is diminished by fixing agents: increased permeability results in haemolysis. All varieties of haemolysis can be explained as due to disintegration of the envelope of the corpuscle.

4. The advantages of the discoid shape are pointed out. The characteristic shape is the result of two sets of forces:

(*a*) tension of liquid surfaces tending to cause a spherical shape;

(*b*) repulsive forces between the dispersed particles of the corpuscular contents.

5. Crenation is briefly described. It is explained as due to protrusion through weak areas in the envelope of the corpuscle.

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XXXI. PEPTISATION OF GELATIN BY MIXED LIQUIDS.

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(*Received December 14th, 1923.*)

Gelatin is dissolved or peptised to form clear, viscous colloidal solutions by a number of organic liquids, taken singly or in mixtures, yet very little work has been done on sols and gels with liquids other than water. The literature concerning the influence of non-electrolytes such as glycerol, alcohol, acetone, etc., on the physical character of the hydrosols and gels of gelatin is also meagre, the main interest having centred around the action of electrolytes.

Ostromysslensky [1907] pointed out that since proteins are complicated amides, it was to be expected that they would prove to be soluble in simple amides and found that the albumoses and peptones dissolve in formamide. S. Levites [1911] also stated that this liquid is a solvent of glutin and Witte's peptone. Formamide is an excellent solvent of gelatin at ordinary room temperatures.

Gelatin swells in glacial acetic acid and on warming forms a fluid sol. It is readily dissolved by crude carbolic and cresylic acids [cited after the First Report on Colloid Chemistry, 1917, p. 46], also in lactic acid of density 1·2.

Retthausen [later ref. 1899] mentioned in 1882, that the proteins from hemp and castor seeds dissolved in moderately concentrated glycerol to form opalescent sols whilst later Osborne found that many proteins were soluble in glycerol after they had been precipitated or crystallised from salt solution.

Liquid substances, in which the hydroxyl or amino group is a predominating feature of the chemical constitution, are usually solvents of gelatin or become solvents with a small quantity of water or acetic acid; thus pure glycerol, phenol, benzyl alcohol, pyridine, etc., are non-solvents, but with the addition of a small proportion of water or acetic acid they become excellent dispersing media. It is possible to add glycerol, aniline, cresol, benzyl alcohol, etc., in large amounts to a concentrated solution of gelatin in water or acetic acid, according to the miscibility, without the development of any opalescence or precipitation of the gelatin, but with other liquids such as benzyl acetate, triacetin, chloroform, epichlorohydrin, etc., a relatively small quantity is sufficient to begin precipitation.

Alcohol, acetone and other organic liquids when added in sufficient quantity cause the precipitation of gelatin from aqueous solution but there is the possi-

bility that in smaller addition the solubility might actually be increased in accord with the well-known observations that a number of proteins, *e.g.* the gliadin of wheat and zein of maize, although soluble in the mixtures, are not peptised by the pure liquids. The degree of solubility varies with the composition; thus Galeotti and Giampalmo [1908] found that the "optimum" solvent mixture of alcohol and water for zein contained about 60 % alcohol.

According to L. des Bancels [1908] gelatin is peptised more readily by aqueous methyl alcohol, ethyl alcohol and acetone, than by pure water in the presence of electrolytes, the effect increasing with increase in the amount of non-electrolyte. On the other hand, Traube and Köhler [1915] state that methyl alcohol under some conditions accelerates the rate of gel formation, suggesting that the solubility is actually decreased by the presence of methyl alcohol. The question whether gelatin is more soluble in aqueous mixtures of the alcohols and acetone than in pure water, is complicated by the fact that gelatin is not a chemical individual but a heterogeneous substance and it usually forms turbid solutions in these aqueous mixtures, indicating incomplete solution or lack of uniform dispersion of the various constituents of the protein.

Extension of the Ordinary Concept of Solubility to the Peptisation of Gelatin.

At first sight there is apparently a difficulty in distinguishing between degrees of solubility of gelatin and similar substances since they appear to be completely miscible with their solvents. This was early recognised by Thomas Graham [1864]. The existence of a saturation concentration, as in the case of a crystalloid, cannot readily be shown, since with rise in concentration the sols become very viscous and intractable whilst any undissolved gelatin is distributed as a swollen, invisible mass. It is possible to precipitate gelatin from solution in the form of a white mass by rapid cooling and stirring, instead of obtaining the usual jelly; this applies in particular to aqueous alcohol or acetone sols.

It was found from a study of the reversible sol-gel transition for systems of cellulose acetate in benzyl alcohol [Mardles, 1923], a finding which is applicable to systems of gelatin, that the scattering of light by a colloidal system is increased markedly during gelation, the effect being a function of time. The coarsest-textured gels were obtained by rapid cooling and stirring, a condition which favoured condensation of the dispersed particles rather than their aggregation into a jelly structure. It is characteristic of a gel to become coarser with age or to develop crystals as in the case of the camphorylphenyl thiosemicarbazide gels of Hatschek [1912].

These observations suggest that gelation is a consequence of the saturation concentration having been exceeded [cf. von Weimarn, 1911] and so the curve connecting the maximum gelation temperature with concentration is analogous to the solubility curve of a crystalloid.

Fairbrother and Swan [1922] have determined the solubility of gelatin in water at temperatures at and below 22° with the following results:

Table I.

Temp. ° C.	Solubility	Temp. ° C.	Solubility
22	1 part per 1000	15–17	0·5 part per 1000
18·3	0·7 part per 1000	0	0·2 part per 1000

The gel from concentration 2 g. per 100 cc. melted at 29°.

Quite recently it has been shown by Knaggs, Manning and Schryver [1923] that by repeated "recrystallisation" of gelatin, a supernatant fluid is obtained which contains about 10 mg. of the solute per 100 cc. of the solvent and this concentration remains constant independently of the strength of the solution from which the gelatin separates and represents the solubility of gelatin in water at the temperature of the experiment.

The stability of a colloidal solution is a function of the degree of dispersion, electric charge on the particle, etc. and the measure of this stability against the precipitation of the colloid by the addition of a miscible "non-solvent," or tendency to set to a gel, affords a method of appraising the solubility. The indirect method of determining the solvent power by noting the quantity of a miscible "non-solvent" required to begin the precipitation of the colloid has been extensively used in connection with the study of cellulose esters and their solvents. Deschiens, Guilleminot and Gault [1919] found that 1 g. of cellulose acetate did not dissolve in 0·5 g. of acetone but the solution in 1 g. of acetone could be diluted with 0·45 g. of water before precipitation occurred [cf. also Sproxton, 1921; Mardles, 1923].

A similar behaviour can be observed with gelatin sols; thus in formamide or acetic acid solution with increase in concentration there is a diminution in the amount of diluent required to begin precipitation (Table II) and at the saturation concentration the solvent power number would reach a minimum or zero value in the case of a true non-solvent.

Table II.

Gelatin in formamide, 25°		Gelatin in acetic acid, 25°	
Concentration, g. per 100 cc.	Vol. in cc. of acetone to begin ppn. in 1 cc. of sol.	Concentration, g. per 100 cc.	Vol. in cc. of chloroform to begin ppn. in 1 cc. of sol.
20	0·75	20	0·5 *circa*
10	0·85	10	0·65
2	0·95	5	0·77
1	1·05	1	0·85
0·5	1·1	0·5	0·98
0·2	1·2	0·2	1·08
—	—	0·1	1·1

The solvent power numbers given later for gelatin in mixed liquids (Figs. 2 and 3) represent the volume in cc. of the diluent named required to begin precipitation of the gelatin in sols of concentration 15/100 at 20°, the larger the amount of diluent added the greater being the solvent power.

With some colloidal substances, notably cellulose esters, the best solvents

yield the least viscous sols and to show that this phenomenon is also applicable to gelatin, viscosity measurements are given.

Hitherto, it has been shown by the experiments of Levites, Pauli and others, that non-electrolytes in low concentrations usually change the viscosity of systems, such as gelatin in water, only to the extent by which they increase the viscosity of the pure dispersion medium.

Experimental.

Materials. Three samples of gelatin, of best quality, were used in the air-dry condition: Coignet's, ash 1·6 %; Nelson's, ash 2·5 and 2·2 %.

Pure liquids were used; their physical constants are given later.

The solutions were prepared by allowing the gelatin to swell in the liquids and then keeping in a warm place with occasional shaking until dissolution was complete. The sols were examined within 36 hours from the time of preparation since slight deterioration usually occurred with time.

Mixtures of Acetic Acid.

Acetic acid and water. Density of acid, 1·0496 25°/4°. Gelatin swelled in the acetic acid and water, each taken singly, at ordinary room temperatures, dissolution being effected by slightly warming. In the majority of cases with the mixed liquids the gelatin dissolved without warming, indicating the higher solvent power of the mixed liquids. With a concentration of 15 g. per 100 cc., when the solutions were cooled to 0°, those containing more than 70 % and less than 90 % acetic acid remained in the fluid state whilst at − 10° only the 80 % acetic acid sol retained its fluidity, the remainder being firm jellies. The "setting point" of the various sols was approximately as follows:

Table III.

	Weight % water							
Composition	100	90	80	60	30	20	10	0
"Setting point"*	32°	28°	23°	15°	0°	− 10°	0°	20°

* From viscosity data and mechanical tests.

These data and the solvent power numbers shown diagrammatically (Fig. 2) indicate that the optimum solvent mixture contains about 80 % acetic acid, a composition approximating closely with the simple molecular proportion, CH_3COOH, H_2O (77 % acetic acid).

The viscosity of a number of different concentration sols has been determined by means of a set of Ostwald viscometers and the results indicate that with the more dilute solutions the viscosity-composition curve is practically parallel with those for the pure liquids. At higher concentrations the peak to the curve becomes exaggerated and a sag also develops, in keeping with the view that the better the solvent mixture the lower the viscosity. The data are given in Table IV (Fig. 1).

The viscosity values for some of the sols were extrapolated from viscosity-time relations, since incipient gelation occurred at 25° with many of the

higher concentrations. With the accumulation of coarser particles there is a slight variability in viscosity with mechanical treatment, etc.: for this reason the viscosity values are expressed to the nearest significant figure, in C.G.S. units.

Table IV. *The viscosity of solutions of gelatin in mixtures of acetic acid and water.*

a, 0·2 g. per 100 cc. Nelson's, sample 1

Compn. weight % acetic acid	Viscosity
98·3	0·0167
87·8	0·0266
83	0·0288
74	0·0279
66·3	0·0265
48·7	0·0221
23	0·016
0	0·0108

b, 0·6 g. per 100 cc.

Compn. weight % acetic acid	Viscosity
98·3	0·02
89·3	0·0318
82·7	0·0342
78·6	0·0355
72·5	0·0345
62·2	0·0295
50	0·0255
35	0·0207

c, 5 g. per 100 cc.

Compn. weight % acetic acid	Viscosity
98·3	0·056
89·8	0·117
79·6	0·136
69·5	0·128
50·2	0·093
30	0·071
15	0·061
0	0·041

d, 10 g. per 100 cc.

Compn. weight % acetic acid	Viscosity
98·3	0·2
90	0·3
80	0·321
60	0·234
30	0·143
15	0·097
0	0·083
—	—
—	—
—	—
—	—

e, 15 g. per 100 cc. Coignet's

Compn. weight % acetic acid	Viscosity
100	1·27
90·4	1·1
80·8	1·06
71	0·96
61	0·69
51	0·62
41	0·44
31	0·41
20	0·4
10	(0·39)
0	(0·38)

f, 20 g. per 100 cc.

Compn. weight % acetic acid	Viscosity
100	4·2
90·4	2·46
85·6	2·4
75	1·75
70	1·7
53	1·15
42	1
30	(0·9)
—	—
—	—
—	—

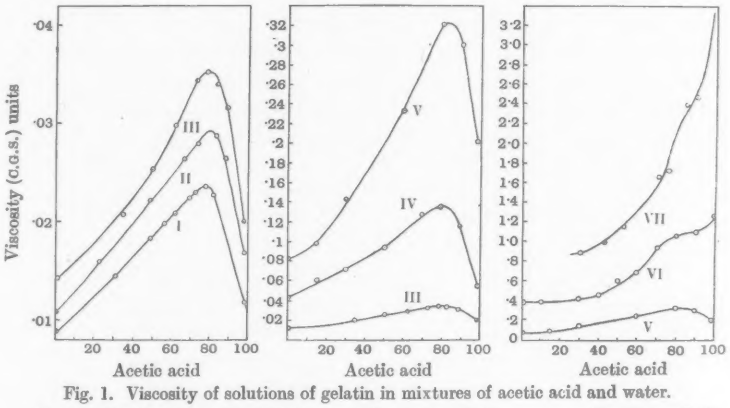


Fig. 1. Viscosity of solutions of gelatin in mixtures of acetic acid and water.

I. Liquids alone [Dunstan 1904].
II. 0·2 g. gelatin per 100 cc.
III. 0·6 g. gelatin per 100 cc.
IV. 5 g. gelatin per 100 cc.
V. 10 g. gelatin per 100 cc.
VI. 15 g. gelatin per 100 cc.
VII. 20 g. gelatin per 100 cc.

The densities of the colloidal sols and gels at 25°, taken to three places of decimals, were found to lie on curves practically parallel to those for the pure solvents.

The solvent power numbers were determined by noting the relative volume of acetone at 25°, required to begin the precipitation of the gelatin, the concentration being 15 g. per 100 cc. The results are expressed in Fig. 2 (p. 221).

Systems of gelatin in water, with the addition of some acetic acid, have been studied by a number of workers with particular reference to swelling. In general the addition of acetic acid increases the swelling [cf. Procter and Wilson, 1916; Procter and Burton, 1916; Kuhn, 1921], it being reported that whereas with strong acids the maximum swelling occurred at lower concentrations, with weak acids it occurred at higher concentrations (Kuhn), and the swelling of gelatin in acetic acid solutions increased with increasing total concentration of acid and was not depressed by the addition of excess (Procter and Wilson). Experiments have been carried out by Bogue [1922] on the influence of acetic acid on the viscosity and jellying power of bone and hide glues.

The increased swelling and the lowering of the gelation temperatures by the addition of acetic acid are in accord with the finding of increased solubility. Levites states that "the more readily gelatin dissolves in a solution the less rapidly will the sol gelatinise"; Wo. Ostwald [1919] has pointed out the close relationship between osmotic pressure, swelling, and viscosity of gelatin solutions. In general with an increase in the degree of dispersion there is an increase in the swelling and osmotic pressure, and a lowering of the viscosity.

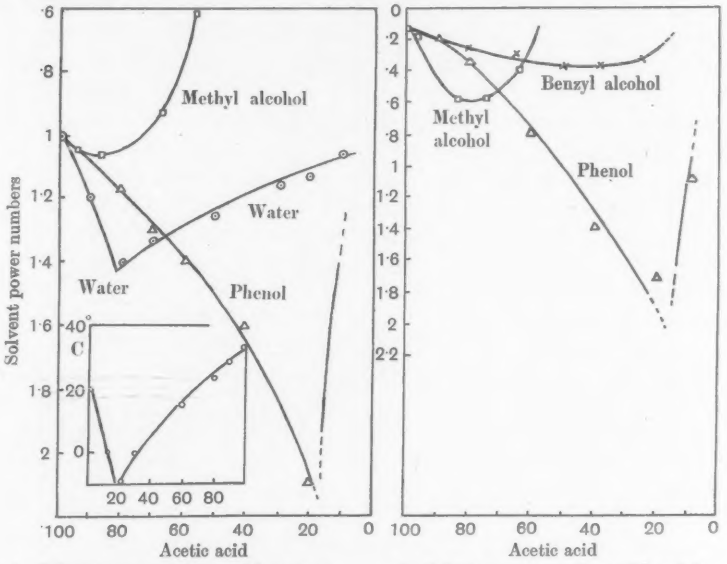
Acetic acid and methyl alcohol. The methyl alcohol used was acetone-free; density at 25°, 0·7875. The density of the acetic acid was 1·0443 at 25°. Gelatin dissolved on warming in mixtures containing up to half their volume of methyl alcohol, and when the solutions were allowed to cool, clear firm jellies were formed. The solubility of gelatin in acetic acid is increased by the addition of some methyl alcohol, but excess forms a non-solvent mixture. The solvent power data are given in Fig. 2. The viscosity values obtained are given in Table V.

Table V.

a, Liquids alone. Temperature 25°		*b*, Conc. 2 g. per 100 cc. Temperature 25°		*c*, Conc. 10 g. per 100 cc. Temperature 35°	
Compn. weight % alcohol	Viscosity	Compn. weight % alcohol	Viscosity	Compn. weight % alcohol	Viscosity
0	0·0119	0	0·0215	0	0·4
9·24	0·0129	3·2	0·0236	6·6	0·36
13·16	0·0129	9·6	0·0275	13·2	0·35
18·6	0·0126	15·6	0·029	18·5	0·356
28·7	0·0115	25	0·0235	32·5	0·356
43·1	0·0097	33	0·0278	43·3	0·42
51·6	0·0091	42	0·0244	—	—
60·2	0·0082	53	0·0165	—	—
79·1	0·0069	—	—	—	—
100	0·0057	—	—	—	—

The curve relating the viscosity and composition for the less concentrated solutions is again similar to that for the pure solvents but with an exaggerated peak, whilst with the higher concentration a sag has developed, supporting the finding of increased solubility in the mixtures. A similar behaviour was observed with mixtures of water and pyridine, etc.; data given later.

Acetic acid mixtures with phenol and benzyl alcohol. It was found possible to prepare solutions containing a high percentage of these liquids which, taken singly, are non-solvents. The solutions remained fluid, on standing for some time at temperatures below 0°, so that the solubility of gelatin in acetic acid has been considerably increased by the addition of these "non-solvents." This is shown in Fig. 2 from the solvent power numbers.



1. Solvent power numbers with acetone. Inset—"Setting points" of acetic-water sols. 2. Solvent power numbers with xylol.

Fig. 2. Relative solvent power of mixtures of acetic acid for gelatin.

Mixtures of Formamide.

Gelatin dissolved readily in formamide, M.P. $-2°$, forming clear sols. It was also possible to prepare solutions in formamide containing a large proportion of phenol and *o*-cresol.

The solvent power-composition curve in the cases of mixtures with water and acetic acid (Fig. 3), indicates increased solvent power beyond the average value, whilst the curve for the phenolic mixtures was sinuous.

The viscosity values given in Table VI (Fig. 3 *a*) show that the curve relating viscosity and composition is similar in shape to that for the pure liquids but greatly exaggerated.

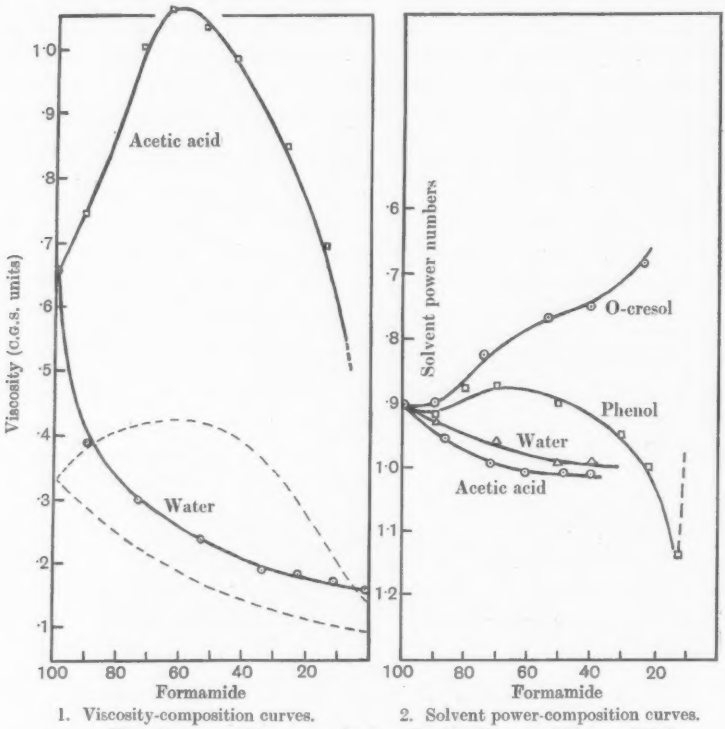
Table VI. *Concentration of solutions.* 10 *g. of Coignet's gelatin per* 100 *cc.* 25°.

(*a*) Formamide and water

Compn. weight % formamide	Viscosity
100	0·67
91·2	0·38
72·8	0·3
53·3	0·24
32·8	0·18
22·2	(0·18)
11·2	(0·17)
0	(0·16)

(*b*) Formamide and acetic acid

Compn. weight % formamide	Viscosity
100	0·67
91	0·74
72·8	1·01
62·2	1·08
52·1	1·04
42	0·98
27	(0·85)
11·9	(0·7)



1. Viscosity-composition curves. 2. Solvent power-composition curves.

---- Viscosity-composition curves for pure liquids [Merry and Turner, 1914].

Fig. 3. Gelatin in mixtures of formamide.

Water Mixtures of (*a*) Pyridine, (*b*) Dimethylamine, and (*c*) Diethylamine.

The mixtures peptise gelatin, on gently warming, even when 50 % of the organic base is present in the case of pyridine and diethylamine, although when taken in the pure state, these liquids are non-solvents. The addition of about 10 % to 20 % pyridine actually increases the solubility of the gelatin but further additions lower the solvent power progressively. This finding is

in accord with that by Levites [1911], who found that although caseinogen and glutin are both insoluble in water and anhydrous pyridine, they dissolve in the mixed liquids.

The addition of pyridine lowers the "setting-point" or gelation temperature, at first, but ultimately raises it again as would be expected from the solvent power data (Table VIII) and from the sag to the viscosity-composition curve.

The jellies containing appreciable amounts of pyridine were thin and easily broken up by shaking or stirring, thus at 8° the relative rigidity of the gels of concentration 10 g. per 100 cc., determined from the angle of twist of the inner of two concentric cylinders between which the gel had set, a torque acting on the inner cylinder, was approximately as follows:

Table VII.

Composition. vol. % pyridine		0	10	30	50
Relative rigidity at	8°	4	0·6	0·3	Small
„	13°	1·9	0·3	Very small	

A set of gels prepared at 10°, several days old, were warmed to 20°: after an interval of an hour those containing less than 20 % pyridine remained as firm jellies whilst those in the neighbourhood of the composition 30–40 % pyridine were mobile, the gel structure having collapsed.

Table VIII. *Viscosity and solvent power data for systems of gelatin in mixtures of water and pyridine.*

Temperature 25°. Nelson's gelatin, sample 1.

Compn. weight % pyridine	Concentration liquids*	g. per 100 cc.				Acetone number
		2	4	10	15	
0	0·0089	0·0145	0·023	0·083	(0·18)	1
10	0·0111	0·0189	0·029	0·103	0·14	1·05
20	0·0134	0·0234	—	0·129	0·22	0·9
30	0·0165	0·0268	—	0·179	0·27	0·7
40	0·0184	0·0298	—	0·185	0·31	0·5
50	0·02	0·0315	—	0·204	0·32	0·3
60	—	0·0307	—	—	—	—

* Values extrapolated from data given by Dunstan, Thole and Hunt [1907].

Table IX. *Viscosity data and acetone numbers. Coignet's gelatin in aqueous mixtures of dimethylamine and diethylamine.*

Concentration 10 g. per 100 cc. 30°

(*a*) Water-dimethylamine			(*b*) Water-diethylamine			
Compn. weight % dimethyl-amine	Viscosity	Viscosity increase during 15 min.	Compn. weight % diethyl-amine	Acetone number	Viscosity	Viscosity increase during 15 min.
2	0·15	—	0	—	0·22	0·4
3·75	0·14	—	2·1	1	0·2	0·1
6·25	0·12	0·03	3·2	1·04	0·17	—
10	0·1	0·005	5·7	1·1	0·12	—
—	—	—	7·9	1·2	0·11	—
—	—	—	11	1·15	0·13	—
—	—	—	23·1	0·93	0·2	—
—	—	—	31·5	0·8	0·22	—
—	—	—	41	0·7	0·26	0·08

Dimethylamine and diethylamine behaved similarly to pyridine by increasing the solvent power of water for gelatin, when added in moderate amounts. Traube and Köhler [1915] found that pyridine, diethylamine and piperidine inhibited formation and accelerated solution of the gel, whilst Fischer and Sykes [1915] showed that pyridine and ethylamine increased swelling.

These findings are in accord with the view of increased solvent power.

Water Mixtures of (*a*) Methyl Alcohol, (*b*) Ethyl Alcohol, (*c*) Acetone, and (*d*) Glycerol.

Gelatin dissolved in the above mixtures, on warming, when the systems contained at least half a volume of water, with the exception of glycerol, with which solutions were obtained with a much smaller proportion of water.

With decreasing amount of water the solutions were more difficult to prepare and visible, insoluble matter accumulated, whilst the sols in the case of acetone and alcohols were turbid, yielding opalescent or milky-white gels on cooling. Hardy [1900] studying the mechanism of gelation in the ternary system, 13·5 g. gelatin, 50 cc. water, 50 cc. alcohol, found the system to be clear and homogeneous above 20° but as the temperature fell below this limit a clouding occurred due to the appearance of fluid droplets which gradually increased in size until they measured 3 μ. On cooling further these fluid droplets became solid and began to adhere to one another.

Fischer and Sykes [1914] have shown that the aliphatic alcohols and acetone depress the degree of swelling of gelatin in water, the greater the concentration of non-electrolyte the greater being the effect.

Table X. *Viscosity data for systems of gelatin in methyl alcohol-water mixtures.*

(*a*) Concentration 5 g. per 100 cc. 28°

Compn. volume % alcohol	Viscosity	Viscosity increase after 30 min.
0	0·058	0·045
10	0·064	0·035
21·5	0·073	0·047
25	0·094	0·047
40	0·096	0·071

(*b*) Concentration 15 g. per 100 cc. 30°

Compn. volume % alcohol	Viscosity	Viscosity increase after 15 min.
0	0·26	0·342
10	0·265	0·345
20	0·52	0·75
30	0·59	1·02
—	—	—

Table XI. *Viscosity data for systems of gelatin in ethyl alcohol-water mixtures.*

(*a*) Concentration 2 g. per 100 cc. 25°
Nelson's gelatin, sample 1

Compn. volume % ethyl alcohol	Viscosity	Viscosity increase after 30 min.
0	0·0155	0·002
5	0·018	0·001
10	0·02	0·001
15	0·022	0·0005
20	0·026	0·001
30	0·029	0·001
40	0·033	0·067
50	0·034	Precipitate visible

(*b*) Concentration 10 g. per 100 cc. 30°

Compn. volume % ethyl alcohol	Viscosity	Viscosity increase after 30 min.
0	0·15	0·02
4	0·17	0·026
10	0·21	0·03
17·5	0·2	0·02
25	0·2	0·023
34	0·28	0·02
40	0·32	High
—	—	—

A set of gels, concentration 5 g. per 100 cc., was prepared at 0°. At 20° the gels containing over 25 % methyl alcohol were milky-white; at 30° the system containing 40 % methyl alcohol was similar in consistency to a mucilage, whilst the systems containing 30 and 25 % methyl alcohol respectively were thin trembling jellies; at 31° a few minutes later only those systems containing less than 10 % methyl alcohol were firm jellies.

The gels of concentration 10 g. per 100 cc. freshly prepared at 17° were translucent and were firm for concentrations up to 17·5 % alcohol, almost mobile up to 34 %, whilst the remainder existed as thin trembling jellies. The system containing 25 % ethyl alcohol had a slightly lower "setting point" than the others, as determined by the fall of a small steel sphere. Bechhold and Ziegler [1905], working with the same concentration systems, found that the addition of 2 mols. ethyl alcohol lowered the "setting point" from 31·66° to 30°.

Although it appears that the "setting point" is lowered by the alcohols, suggesting increased solubility, there is no evidence from the viscosity data that such is the case, at least to any marked extent.

Simon [1907] studied the effect of alcohol on the increase of viscosity with time in albumin solutions, and found that the rate of increase became much larger as more alcohol was added, especially when the alcohol content exceeded 26 %. This rate of increase of viscosity with time, $\Delta\eta/\Delta t$, has been used by von Schroeder [1903] as a method of noting the tendency to gelation of aqueous gelatin solutions, and from the last column, Table XI, which gives the viscosity increase over an interval of time, it will be seen that there is no decided indication from this method that gelation is inhibited by the addition of alcohol.

An explanation of the phenomenon is afforded from a perusal of the data relating to the approximate rigidity of the jellies containing alcohol. It will be noticed that the temperature coefficient of the rigidity is lowered considerably by the addition of alcohol.

Table XII. *The relative rigidity of hydrogels of gelatin, with the addition of (a) ethyl alcohol, (b) acetone.*

Concentration 10 g. per 100 c.c.

	(*a*) Vol. % alcohol				(*b*) Vol. % acetone			
Temperature	0	10	25	40	10	20	30	40
8	4	1·7	2·2	1·9	—	0·9	1·7	1·5
10	3·2	—	1·5	—	2·7	0·7	1·1	1·2
12	2·2	0·8	1	0·9	2·1	0·5	0·8	0·8
16	1	0·5	0·6	—	1·1	0·25	0·45	0·35
20	0·4	0·25	0·3	0·1	0·55	0·1	0·05	0·05
22	0·2	0·1	0·13	0·04	0·25	0·05	—	—
24	0·1	—	0·02	—	0·1	—	—	—

These results suggest that there is no actual depression in the gelation temperatures, or if so, very little, since the curves relating rigidity and temperature meet the temperature axis about the same place.

It appears that although the addition of alcohol might increase the solubility of a proportion of the constituents of gelatin affecting the rigidity of the gels, yet another proportion suffers in solubility when about 20 % alcohol is present and it is this portion which tends to form a gel structure or is actually precipitated. This view is supported by the fact of the coarse structure of some of the gels (opalescent or milky-white), the presence of a sediment and the increasing difficulty in peptising all the gelatin with rise in alcohol content.

In general, the addition of alcohol up to about 30 % does not decrease the solubility of the main constituents of gelatin in water to any appreciable extent, and the smaller additions might even appear actually to increase the solubility, but against this a minor portion of the constituents is not so readily peptised by the mixtures of water and alcohol. Above 40 % the addition of alcohol rapidly decreases the solvent power.

The same behaviour is observed with the dissolution of gelatin in mixtures of water and acetone. The "setting point" is apparently lowered by the addition of acetone since this substance reduces considerably the rigidity of the gels, but the viscosity data shows that gelation is not actually inhibited.

Table XIII. *Viscosity data for sols of gelatin in mixtures of water and acetone.*

(*a*) Concentration 10 g. per 100 cc. 35°		(*b*) Concentration 15 g. per 100 cc. 30°		
Volume % acetone	Viscosity	Volume % acetone	Viscosity	Viscosity increase after 15 min.
0	0·08	0	0·26	0·08
10	0·096	10	0·34	0·09
20	0·106	20	0·4	0·11
30	0·103	30	0·41	0·1
40	0·117	40	0·51	0·18

The influence of glycerol on the mechanical properties of the hydrosols and gels of gelatin has been studied by a number of workers. Bechhold and Ziegler [1905] showed that the M.P. of the gels is raised; 10 g. per 100 cc. hydrogel had M.P. 31·66° but with the addition of 2 mols. of glycerol it was 32·17°. Lillie [1907] found that the osmotic pressure of a gelatin hydrosol was lowered from 6·2 mm. of Hg to 5·9 by the addition of $M/6$ glycerol. Arisz [1915] has studied the reversible sol-gel transition in a glycerol solution, sp. gr. 1·176, corresponding to about 32 % water. At 50° with a 10 % gelatin solution, repeated passage through the viscometer lowered the viscosity, suggesting the presence of a "structure" formed from undissolved or coarsely-dispersed material.

According to Michaud [1922], the rigidity of hydrogels of gelatin and gelose is not substantially altered by the addition of glycerol.

It was found that the glycerol solutions peptised gelatin on warming to form clear sols, but when the proportion of water became small higher temperatures and longer time were required.

The glycerol-rich solutions, say about 90 %, peptised with difficulty, but the sols could be obtained by preparing the hydrosol first and then adding the requisite quantity of glycerol.

A number of viscosity values at 25° (incipient gelation tended to increase the values, whilst the water associated with the air-dried gelatin tended to lower them), obtained with a concentration of 2 g. per 100 cc., indicated that the viscosity-composition curve was parallel to that for the mixed liquids. At 25° the rate of change of viscosity with time for the sol containing 80 % glycerol was about double that for the hydrosol.

In general, from the lowered osmotic pressure, raised melting point of the gels, tendency to gelation, etc., it appears that glycerol does not increase the solubility of gelatin in water at first, at least to any marked extent, but decreases it slowly, the effect increasing as the glycerol content rises until in the neighbourhood of 90 % glycerol, the capacity for peptising practically ceases.

The Surface Tension of some Sols of Gelatin.

Gelatin lowers the surface tension of water against air, even when added in small amounts, and the lowered surface tension is a function of the age of the sol and mechanical treatment, on account of the gelatin concentrating in the surface, in accord with the theorem of Gibbs. Reynolds [1921] found that with a 0·5 % gelatin hydrosol the value for a fresh surface was 71·1 dynes per cm., but after 24 hours this fell to 65·5.

It is of some interest to inquire whether the lowering of the surface tension of the dispersion medium is a universal property of gelatin at different temperatures. The following data show that this is not the case, but that with some liquids the surface tension is actually raised. With water, formamide and acetic acid the surface tension of the pure liquid is lowered considerably, whilst in the case of mixtures of acetic acid with 20 % water or with an equal weight of phenol or *o*-cresol the tension is slightly raised.

There appears to be some relation between the degree of dispersion and the surface tension. Walker [1921] working with the salts of fatty acids, and Duclaux [1878] with aliphatic alcohols, found that the power of lowering the surface tension of water rises to a maximum with increase of molecular weight in a homologous series, so that the largest particles depress the most. From this conclusion the size of the dispersed particle of gelatin in water, formamide or acetic acid is greater than that in the phenol mixture, which is a possibility considering the large solvent power numbers of the phenol mixtures compared with those of water, etc. On the other hand, the addition of alcohol or acetone to the gelatin hydrosols raises the tension until it is equal to or greater than that of the dispersion medium, and there is no reason to believe that the degree of dispersion is increased. This may, however, be explained on the ground of decreased solvation so that though the degree of dispersion be actually decreased by the addition of alcohol or acetone the actual size of the solvated particle might be smaller.

Table XIV. *Surface tension measurements obtained with the use of Sugden's* [1924] *apparatus, maximum bubble-pressure method.*

Freshly prepared solutions were used since the surface tension varied with age.

(*a*) Gelatin in water, 5 g. per 100 cc.

Temperature	Dynes/cm.
20	67·2
25	66·1
33	64·5
41	62
50	60·3
60	59
75	55·9

(*b*) Gelatin in acetic acid, 5 g. per 100 cc.

Temperature	Dynes/cm.
10	25·7
22	24·9
27·5	24·4
45	23·6
61	22·7
73	21·9
—	—

(*c*) Gelatin in formamide, 5 g. per 100 cc. Results varied with shaking, etc

Temperature	Dynes/cm.
9	62·1
15	56
18	61·2
23	58·3
36	56·7
38	57·3
40	55·9
59	56·7

(*d*) Formamide alone

Temperature	Dynes/cm.
10	61·2
15	60·5
31	59·1
54	57·4
59	56·9
—	—
—	—
—	—

(*e*) Gelatin 5 g. per 100 c.c. in phenol-acetic acid (density of acid at 25° 1·063). 50 % weight

Temperature	Dynes/cm.
10	37·3
15	36·5
26·5	34·4
45	33·3

(*f*) Phenol mixture, no solute

Temperature	Dynes/cm
14	36·9
26	35·9
35	34·6
—	—

(*g*) Gelatin, 5 g. per 100 cc. in a mixture of equal parts *o*-cresol and acetic acid

Temperature	Dynes/cm. No solute	Dynes/cm.
10	34·3	—
20	33·3	33·9
35	31·6	32·2
50	30·1	30·5
60	29	29·4

(*h*) Gelatin, 10 g. per 100 cc. in water-ethyl alcohol. 30°

Composition weight %	Dynes/cm.
0	60·8
4	53·6
8	47·4
12·3	42·2
24	31·5
33	29·9
44·4	29·1

(*i*) Gelatin, 5 g. per 100 cc. in 80 % acetic acid. 27·5°. 33 dynes/cm.
No solute 31·9 „

The surface tension, by this method, of acetone-water solutions of gelatin containing 10 % and more of acetone was found to be practically the same as that of the dispersion medium.

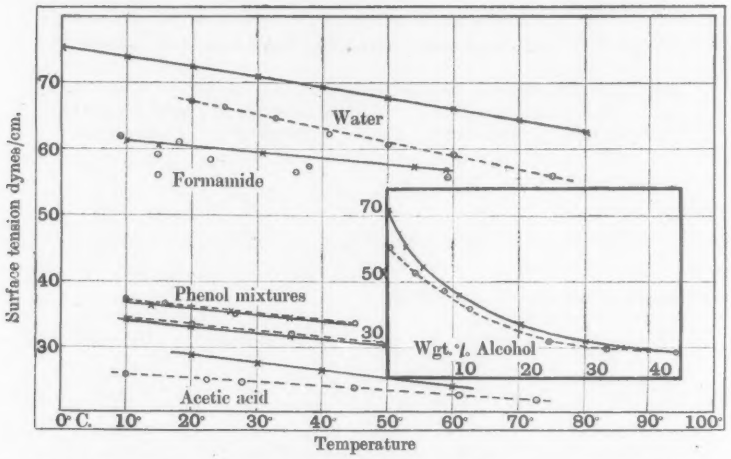


Fig. 4. The surface tension of the pure dispersion medium (————) and gelatin sol (- - - -) at various temperatures. Inset—Surface tension-composition curves for pure liquids and sols in water-ethyl alcohol mixtures at 30° C.

Conclusions.

The results of the experimental work presented indicate that the solubility of gelatin is greater in the mixed liquids than the average value, and under some conditions the solvent power of water can be increased, or not appreciably diminished, by the addition of a non-solvent such as pyridine, glycerol, alcohol, etc.

A precise knowledge of the process of swelling is needed for the explanation of this phenomenon, since dissolution of the gelatin is preceded by its swelling and it is the swollen colloidal material which is actually dispersed. The recent researches of Knoevenagel and co-workers on the nature of swelling, indicate that it is a process in which the laws of constant combining proportions hold and is of the nature of a chemical combination, a conclusion in keeping with the view that the solvated particle is an individual chemical complex and not merely a physical molecular aggregate.

The reciprocal attraction between the molecules of the dispersion medium and those of the colloid must depend upon their relative specific characters and when the attraction is high there must be a consequent large fall in the force of cohesion between the colloidal particles (a force tending to resist dispersion), since these are forced apart during the process of swelling. The addition of a non-solvent such as alcohol does not simply dilute the system or act as a mere dehydrating agent, but the alcohol molecules enter into the colloidal complex, altering its size and specific character in general.

Many of the liquids used in the experiments, such as water, or acetic acid, do not exist entirely in the unimolecular form at ordinary temperatures, but

contain molecular complexes or polymers. On mixing the liquids the degree of association of similar molecules would probably be reduced with the simultaneous formation of molecular associates of dissimilar molecules, not necessarily larger or less attractive to the colloid than the original ones, and since this dissociation and the formation of solvated colloidal particles are molecular processes it is not surprising that the optimum solvent mixture should occur in the neighbourhood of that of a simple molecular proportion, a coincidence frequently observed. Although the existence of chemical complexes in many of the binary mixtures of liquids might be definitely proved, any conclusion that the complex is the intrinsic solvent of gelatin cannot be maintained since the solubility is the resultant of a number of factors. In general any factor which tends to a simplification of the molecular condition of the dispersion medium causes an increase of the solvent power with a corresponding higher degree of dispersion and solvation of the gelatin particles; there is also a fall in the viscosity and increase in surface tension of the sols.

Acknowledgment is made to the Royal Society Government Grant Committee for help towards expenses of work with non-aqueous colloidal systems, of which this paper forms a part.

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XXXII. NOTE ON THE SEPARATION OF A FAT PIGMENT FROM ACCOMPANYING FAT.

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(*Received August 3rd, 1923.*)

The very small concentration of pigment substance in adipose tissue fat has always been the chief factor militating against a concise characterisation of its chemical properties, and the various methods devised for its isolation have been built on a very insecure basis of solubility differentiation from accompanying bodies nearly related in chemical properties.

In considering the problem of fat pigment extraction, one recalls in another direction, the work of Noël Paton and others [1898] on the pigments in the muscles and ovaries of the salmon. Here, the pigment-binding substance, because of its protein nature and its marked difference in chemical properties from the fats, phospholipins and pigment itself, was easily freed from pigment; but adipose tissue pigment—adipochrome, as we may call it—is soluble in practically all the fat solvents, and its separation must ultimately be determined by a decided difference in affinity with a common third substance. This method of separation which I have found very successful in the results it yields will be referred to later.

There are various mechanical means of separating pigment in a tolerable state of purity. In this laboratory, a process of fractional sedimentation in light petroleum was attempted and yielded small amounts. The fat was dissolved in the petroleum and allowed to stand for some time when a sedimentation of brownish-yellow substance took place. The supernatant liquid was removed and fresh portions of pigmented fat were introduced, the solution being once again submitted to sedimentation. Finally the sedimented substance was collected, redissolved in light petroleum and allowed to stand for some time. Sedimentation set in again, and this process of sedimentation and re-solution was carried out until a small sample of pigment almost free from fat was obtained. The disadvantage of this method is that because of the close agreement in the solubilities of adipochrome and fat in light petroleum only a small percentage separation takes place.

Another method attempted was to filter a fat emulsion through a Chamberland candle under a pressure of 150 lbs. per square inch. The earlier filtrates contained a completely colourless fat, but the difficulty experienced in the separation of residual pigment from fat was so great as to render this process

practically valueless. In fact, the final extraction by this method merely resolved itself into an operation of fractional sedimentation the chief advantage of which is that it gives a much higher concentration in pigment as compared with fat than the last method.

Such modes of separation as above outlined refer solely to *in situ* extraction of pigment. More efficient methods depend on hydrolytic treatment of the fat.

The common hydrolytic agents used are caustic soda and caustic potash in alcoholic solution. These agents may be quite efficient for ordinary laboratory work, but the chief defect attendant on their use is that the alkali reacts with alcohol producing in very low concentration a yellowish-red substance (probably a mixture of aldehyde resins) similar in appearance to adipochrome in alcoholic solution. This substance is quite as elusive as adipochrome in its behaviour towards solvents. It is soluble in ether, alcohol, cold and warm benzene, light petroleum, chloroform, carbon tetrachloride, in fact, in all the common fat and adipochrome solvents.

Its presence, then, along with extracted pigment tends to render valueless any fundamental tests, since from the ease with which it is formed it may prove a very active agent in the modification of any of the characteristic reactions of adipochrome.

In the lime process of saponification, the fat can be hydrolysed in a very short time if the autoclave is used. For a fat of molecular weight 890 the amount of lime required to convert the glyceride into glycerol and the calcium salt of the fatty acid is 9·4 % of the weight of glyceride, but in order to complete the hydrolysis under ordinary pressure 12–14 % by weight of lime is required. Lime, however, can be used as a catalyst if the conditions of carrying out the process are modified. At a pressure of eight atmospheres complete hydrolysis can take place with a third of the theoretical quantity of lime required to yield the calcium salt: at twelve atmospheres as small a quantity as 1 % by weight of lime is sufficient to effect complete hydrolysis.

The calcium oxide and the baryta methods were attempted here. It was thought that the Twitchell method (French Patent, 456956) while being of considerable use in the process of commercial hydrolysis [see Henderson, 1919] might in the laboratory give rise to undesirable coloured substances and difficultly separable compounds. This method has not meantime been adopted. The calcium oxide and barium hydroxide methods, however, promise to give good results. Practically, the better results were obtained with calcium oxide. The hydrolysis was quite effective with this compound and the small proportion of calcium salt formed was easily disposed of. In the barium hydroxide method, the glycerol-containing portion of the hydrolysate was found to be quite rich in barium salts of the fatty acids present. These came down easily, but not completely on cooling, bringing pigment substance with them, and its separation was rendered the more difficult because of subsequent acidification.

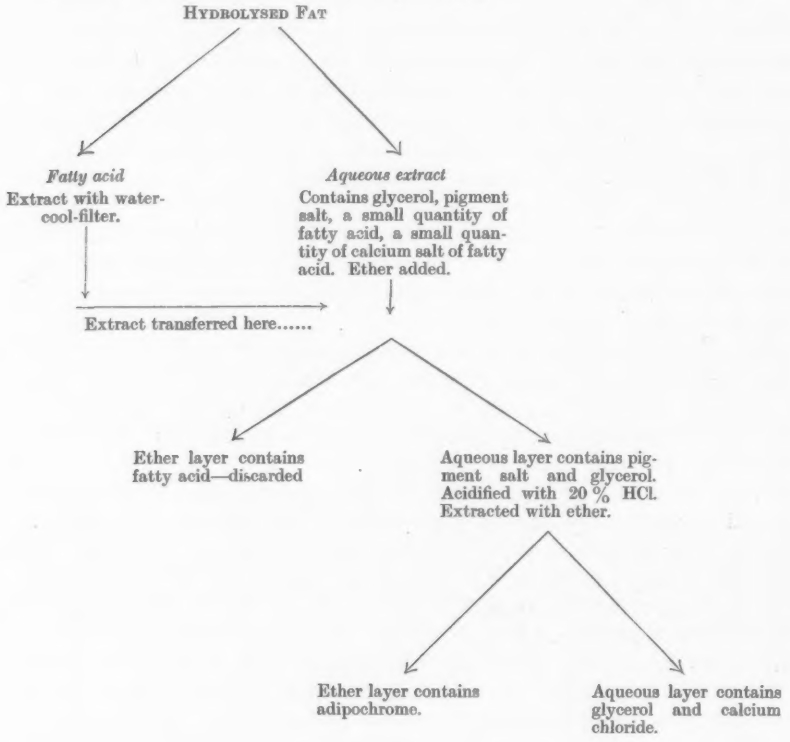
Method.

The method devised for the separation of pigment is illustrated in the following experiment. 200 g. of fat (obtained from the abdominal wall of stall fed cattle) were taken in a 5 litre beaker. About 14 g. of lime were finely powdered in a mortar and intimately mixed with the glyceride. The fatty charge was then covered with distilled water; a piece of well-washed and dried muslin cloth was tied over the mouth of the beaker which was then lowered into the autoclave. A pressure corresponding to a temperature of 140° was maintained for ten hours, and at the end of that time complete hydrolysis had taken place. The liquid subjacent to the fatty acids was removed (through muslin cloth tied to mouth of beaker), filtered, and was found to be straw-yellow in colour. Further extractions were made from the fatty acid with hot distilled water until the extracts were no longer coloured. These were added together in a large beaker and after water had been added were concentrated to a small bulk. A large quantity of water was used in order to dissociate as far as possible the calcium salts of fatty acids present in small quantity in the glycerol-containing aqueous extract. The concentrate was distinctly alkaline in reaction to litmus and in its alkaline state was extracted with ether. The ether layer contained very small quantities of fatty acid dissolved out in the long continued extraction. The yellow pigment substance was entirely taken up by the aqueous layer as it was quite insoluble in ether. The aqueous layer was then drawn off into a second separating funnel and the top ethereal fatty acid-containing layer was discarded. In this way pigment was separated from fatty acid. The aqueous layer still contained glycerol. This yellow layer was acidified with 20 % hydrochloric acid. There was very little change in colour on acidification, but after ether had been added and the contents of the separating funnel shaken up, the yellow colouring matter was completely transferred to the top layer.

In this way the pigment was freed from glycerol. These two simple operations effect a separation of pigment from both fatty acid and glycerol, and the difficulties associated with the other methods of hydrolysis, *e.g.* (1) the development (with alcoholic caustic potash) of an independent coloured substance which manifests the same physical peculiarities as regards solubility as the adipochrome, and (2) the almost inseparable association of adipochrome with fatty acid on account of their near affinity for the common fat solvents, were completely eliminated. It was surmised that adipochrome behaved as an acid substance and was stronger, *i.e.* dissociable to a higher degree than stearic or palmitic acid. It seems to form a stable union with barium hydroxide, calcium hydroxide, generally with the alkaline hydroxides and several other metallic oxides. Calcium hydroxide effects a rather stable union with the adipochrome "acid" substance. This stable union resists hydrolytic dissociation to a marked degree. For example, the barium and calcium salts of stearic acid (insoluble in water) undergo dissociation to such

an extent that they give up their bases on washing with water. The above fact bears on this method of extraction, as the pigment "salt" is retained in the first water layer along with the calcium hydroxide from the broken down calcium stearate while the stearic acid present in very small quantity goes to the ether layer. In this way adipochrome "salt" is separated from fatty acids and their calcium salts. It is soluble in water and in alcohol, but is insoluble in ether, as shown above. On acidification with 20 % hydrochloric acid, or even 10 % tartaric acid solution there is an immediate migration of pigment substance to the ether layer showing that adipochrome which has been freed from combination with calcium is soluble in ether.

The scheme of separation is shown below. The chemical nature of adipochrome will be discussed in a subsequent paper.


Hydrolysed Fat
Fatty acid
Extract with water-cool-filter.
Aqueous extract
Contains glycerol, pigment salt, a small quantity of fatty acid, a small quantity of calcium salt of fatty acid. Ether added.
Extract transferred here.....
Ether layer contains fatty acid—discarded
Aqueous layer contains pigment salt and glycerol. Acidified with 20 % HCl. Extracted with ether.
Ether layer contains adipochrome.
Aqueous layer contains glycerol and calcium chloride.

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XXXIII. THE LIPOCHROME OF ADIPOSE TISSUE IN MALIGNANT DISEASE.

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(*Received August 3rd*, 1923.)

Introduction.

In spite of the fact that in the course of the last ten years a considerable amount of research work has been carried out on the substances pigmenting fatty and other tissues of the body, there still seems to be a lack of unanimity regarding their chemical nature and function.

Thudichum [1869] who was the first to study the chemical character and distribution of these so-called lipochromes in the body, came to the conclusion that the "luteine" which he had isolated as a crystalline substance of a fatty nature, was responsible for the pigmentation of the corpus luteum and egg yolk. He also observed that this compound was to be found firmly held by protein substance in blood serum, in the cells of adipose tissue, and in the yellow fat of the secretion of the mammary gland or butter. In mammals it occurs abnormally in ovarian cysts and in serous effusions; in the plant world, he asserted it was present in seeds such as maize, in the husks and pulp of fruits such as anatto, in roots such as carrots and in the stamina and petals of a great variety of flowers of which he gave a list in his interesting paper. While Thudichum's contribution was of great importance in opening a new avenue of research, the term by which he designated or grouped together the various pigments worked on is rather unfortunate, being one rather of generic than of specific significance as it may be inclusive in many of his own instances of such widely differing substances as carotene, the *L*-, *B*- and *Y*-xanthophylls of Schunck [1903] and the anthocyanin and flavone flower pigments [Dufour, 1887; Devaux, 1888]. Later investigators, notably, Schunck, Escher, Willstätter, Newbigin, Palmer and Eckles, have helped considerably to make our knowledge of the lipochromes more definite.

Newbigin [1898] has isolated a typical lipochrome from the muscles and ovaries of the salmon. The pigment of cow fat does not, however, give the common lipochrome reactions according to this investigator.

Palmer and Eckles [1914] in a series of studies on cow milk and fat pigmentation came to the conclusion, on the other hand, that carotene and xanthophyll were invariably associated together in the pigmented localities,

and surmised that the pigment of human fat was derived exogenously from vegetable food and was of a carotinoid nature.

The present paper is chiefly concerned with the pigment of human adipose tissue, and I shall refer to it as adipochrome in order for descriptive purposes to differentiate it from such pigments as occur physiologically in the corpus luteum, in the epithelium of the seminal vesicles, testicles and epididymis, in the Kupffer cells of the liver, and pathologically in renal epithelium and in cases of brown atrophy of the heart.

There is much difference of opinion regarding the constitution of fat pigment. Some investigators identify it with carotene in spite of the fact that it does not give the lipocyanin reaction with concentrated sulphuric acid[1]; others state that it contains a carotene-xanthophyll complex, while a third group maintains that many fat pigments accumulated under pathological conditions are merely "waste pigments" (lipofuscins) of non-vegetable origin and are closely related in structure to the fat-like substances from which they are held to be derived.

Scope of Research.

The purpose of this research was to determine if the pigment of circumcarcinomatous fat was in any way different from that of normal fat, and in view of the divergence of opinion expressed regarding the constitution of normal fat pigment, it became necessary either to investigate the nature and properties of adipochrome, or to compare, without elucidating its structure, certain of its outstanding chemical properties with those of the pathological fat pigments worked on. I have not carried out an extensive investigation on the structure of adipochrome, although an empirical formula and certain information relative to its chemical constitution are given. A rigid comparison of chemical and physical properties was made not only between pigment from normal and pathological fat, but between these and carotene and xanthophyll from plant sources, and the results are incorporated in this paper.

Experimental.

Isolation of adipochrome.

The pigment-containing fat was obtained—post-operative and post-mortem—chiefly from the breast and abdominal wall. Adipochrome was isolated by various methods, *e.g.* by the well-known saponification method, and by a process outlined by the author in a separate paper [1924] which seemed to possess some advantages over the potash method, which tends to produce resinification of pigments and condensation products of certain impurities present in traces in the alcohol. The aldehyde resins produced from alcohol and possibly from fatty acids are highly coloured, inert substances, closely

[1] Carotene on treatment with concentrated sulphuric acid gives a blue colour and small blue crystals of lipocyanin are formed.

simulating adipochrome in their chemical behaviour and their presence with the latter substance may modify to a considerable extent its characteristic response to chemical or physical agents.

Some properties of adipochrome.

On account of the extremely small quantities of adipochrome isolated, the tests performed were mainly of a qualitative rather than of a quantitative nature, and, in consequence, the accumulation of data of value in the interpretation of function was considerably limited in scope and application.

The elemental composition of the pigment was first ascertained. It was found that carbon and hydrogen were present. In some cases there was a very faint trace of iron, but this was subsequently found to be present as an impurity. Nitrogen was found absent (by Castellana's modified method); phosphorus was also absent (Neumann's test). There was no indication of the presence of calcium or of halogens. Sulphur was absent.

The absence of phosphorus and nitrogen indicates that there is no lecithin residue or structural unit in adipochrome. The closely associated cholesterol is also absent as the substance gives no colour response to the Liebermann reagent.

The pigment as obtained from the sources previously mentioned was a reddish brown paste with a heavy sweat-like odour. It was difficultly crystallisable. On making a slide preparation with alcohol a magma of acicular crystals was obtained. The melting point of fat pigment isolated from a carcinomatous lesion was 35·5°.

An aqueous calcium chloride solution of pigment was decolorised by bromine water. Further confirmatory evidence of the unsaturated nature of the adipochrome molecule was forthcoming on making an osmic acid slide preparation, when a characteristic blackening took place.

The substance under investigation seemed to be of a weakly acid nature. An aqueous alcoholic solution after a very long interval produced a slight colour change towards the red in blue litmus paper.

The original calcium "salt" of adipochrome is insoluble in ether, light petroleum, benzene, chloroform, chloroform + 1 % alcohol (slight purplish colour developed in alcoholic chloroform layer), toluene, carbon tetrachloride, nitrobenzene, quinoline, ethyl alcohol and amyl alcohol. It is soluble in glycerol, ethyl benzoate, acetic anhydride and pyridine.

With selenic acid, formic, citric and tartaric acids, a slight "urochrome" blush is obtained.

Solubility tests were carried out on a porcelain plate with small quantities of adipochrome.

Adipochrome is soluble in aniline, carbon disulphide, ethyl alcohol, amyl alcohol, light petroleum, toluene, benzene, ether, nitrobenzene and carbon tetrachloride.

In concentrated sodium hydroxide it is comparatively easily soluble, a brown

solution which is unchanged by acetic acid, in contradistinction to that of the anthocyanins, being obtained. It is also soluble in concentrated ammonia, concentrated nitric acid and concentrated sulphuric acid. With the latter solvent no violet coloration is obtained[1].

The fat pigment is more easily soluble in ammonia than in caustic soda and a slightly darker brown solution is obtained. When potassium ferricyanide is added to this ammoniacal solution, an immediate colour change to yellow is observed, and on testing this yellow substance with ferric chloride and concentrated hydrochloric acid a deep Prussian blue colour is obtained, showing that in the presence of alkali the pigment substance is able to convert ferric iron to the ferrous state. It acts, therefore, in the presence of alkali as an oxygen distributor of singular potency.

The sulphuric acid solution of pigment on the addition of yellow potassium ferrocyanide shows an immediate colour change to olive green.

Adipochrome is easily precipitated from alkaline solution by tannic acid.

When Cole's solution (copper sulphate and glucose) was added to an ammoniacal solution of pigment there was a retention of the blue colour, but this does not necessarily mean that there is no carbohydrate structure in the substance, since the retention is primarily due to the formation of the cuprammonium ion. By other methods, however, *e.g.* the osazone microscopic method, it was found that there was no sugar group present.

Adipochrome gives (1) no colour reaction with ferric chloride, (2) is not bleached by sulphur dioxide; (3) gives no odour of benzene on heating with soda lime, thus implying the absence of an aromatic group; (4) gives a slight saturation action with zinc dust; (5) does not react with sodium bisulphite. When suspended in a 5 % solution of cane sugar, it gives a very faint pink colour to the solvent after several days.

A characteristic reaction is with H_2SO_4, menthol and alcohol. Equal quantities of acid and alcohol are placed in a test tube containing a single crystal of menthol and a small quantity of adipochrome. Immediately a deep orange, almost red, colour is produced. In a control with the same reagents without adipochrome, a deep rich pink is produced, but only after a comparatively long interval. In the first tube, the colour development is instantaneous and progressive.

Cotte [1903] believes that adipochrome contains a cholesterol group, but we have not found the presence of this group made manifest either by the Liebermann test or by means of the Kahlenberg anhydrous arsenic chloride reagent [Kahlenberg, 1922].

The chemical nature of lipochrome.

It has been remarked by some observers [Palmer and Eckles, 1914] that the fat pigments of adipose tissue are of a carotinic or xanthophyllic nature, and many interesting conclusions regarding their biological significance have

[1] See footnote, p. 236.

been developed from the primary assumption. In the literature we have consulted on the subject there is very little direct reference as to the manner in which the ascription has been made.

The characteristic test for carotene is the development of a blue colour with concentrated sulphuric acid. It also forms a deep violet addition compound with iodine and gives a characteristic spectral absorption in the blue and the violet. It melts at 174°.

Xanthophyll gives the sulphuric acid reaction, forms addition compounds with the halogens, loses its colour on exposure to air and gives a characteristic three band absorption in the blue. It melts at 173°.

Fucoxanthin agrees very closely in chemical and physical properties with the members of the carotene group. It has a melting point 159°.

The evidence adduced in the previous section of this paper is sufficient to show that fat pigment is not of a carotinoid nature. The members of the carotene family have high melting points, ranging from 159° to 174°. Adipochrome has an extremely low melting point, *i.e.* 35·5°; the latter substance is crystallographically indefinite in its structural configuration giving a magma of acuminate crystals occasionally embedded in spheroidal or ellipsoidal aggregates of the same substance, while the carotenes crystallise regularly in the rhombohedral system.

Carotene is soluble in chloroform, carbon disulphide, benzene, difficultly soluble in light petroleum and ether. It is almost insoluble in cold alcohol, but goes into solution on prolonged boiling. Its behaviour is similar with methyl alcohol. Xanthophyll follows carotene closely in its solubility behaviour.

Adipochrome, on the other hand, is comparatively easily soluble in the solvents above enumerated. Even in light petroleum its solubility coefficient is so high as to render its complete separation from binding fat a matter of extreme difficulty.

Adipochrome like the carotenes contains carbon and hydrogen only and is an unsaturated compound. One would then expect that because of its unsaturated nature it would be peculiarly liable to undergo chemical change on exposure to air. On standing, however, there is no progressive increase in absorption of iodine and there is no evidence of autoxidation. It is, therefore, *in vitro*, particularly inert, though possibly in the body it lends itself to ready change in response to specific stimuli. Carotene on exposure to air undergoes chemical change and loses colour; adipochrome is also slightly chromolabile, but, on the other hand, the tendency is towards an increase of intensity of colour on exposure. This increase in colour intensity was noticeably in evidence in the case of a sample of pig fat.

The pigment of pig fat.

The fat, which was removed from a tumour-bearing animal assumed, on straining, a greenish tinge, probably due to a chlorophyllic break-down product. The freshly deposited fat did not seem to contain much pigment. It was poured into a beaker and allowed to stand for several

weeks, when at the circumference of the fat disc formed, deeply embedded in the fat substance and as far removed as possible from the influence of surface oxidation, a zone of reddish brown pigment was observed. There was a gradual shading away of pigmentation towards the fat surface, and at a certain point on the circumference of the ring (on the under side), where there was ample macroscopic evidence of fungal attack, the pigmentation was most intense. The mould was the common *Penicillium glaucum*. Moulds and bacteria are known to produce pigments which are classified on a differential basis of solubility in water and alcohol [Muir and Ritchie, 1919]. The pigment here produced did not give the lipocyanin reaction and was in other respects identical with adipochrome. It would seem that the pig fat pigment is endowed with an inherent chromolability. Micro-organisms may influence the chromogen or pro-chromogen. We would, however, think that the mould depends in the main on fat for its energy supply, and that it employs the already present adipochrome for a subsidiary purpose.

This view is partly borne out by the fact that the pig fat situated in a region of high *Penicillium* concentration is differently saturated from that of the remote areas where the mould distribution is on a sparser scale. The former fat (containing fat pigment) is harder, *i.e.* has a lower iodine value or is more saturated and is less soluble in ether. Adipochrome may possibly be used by the mould as a director of synthesis from a fatty substrate. With pig fat, fatty acid *saturation* is accompanied by increased adipochrome production.

The synthetic activity of the mould in manufacturing adipochrome is not of a very high order. In pathological fats, *e.g.* in cancer, the increase in pigmentation in fat areas near the growth is not so much ascribable to an increase in the synthetic activity of some specific enzyme as to the change in the solvent glyceride-, or free fatty acid-containing pigment. The concentration of oleic acid is increased in circum-carcinomatous fat, and there is a corresponding increase in the solubility of adipochrome. The phenomenon of increased pigmentation in the fat round cancerous areas goes hand in hand with that of increased fluidity of the pigment-containing fat. In this case *desaturation* activity is also accompanied by increased adipochrome production.

Absorption spectra of adipochrome.

The absorption spectra of the adipochrome from various sources further emphasise the difference between it and carotene and allied compounds. The spectrograph used for spectral examination was the Heele instrument, with a three prism train of uviol glass, and the light source was a copper arc. Table I (below) gives the necessary data for constructing a spectrum map for the instrument.

Table I.

Scale reading	10·1	10·4	12·4	13·45	15·3	21·25	28·6	29·4	37·8
$\lambda\lambda =$	6708	6409	5896	5700	5351	4608	4062	4023	3650

The nature of the absorption of adipochrome (in this instance, from a case of fibro-adenoma) is shown in Fig. 1 (Plate I). There is no selective absorption in the blue or violet region of the spectrum. In this plate, there seems to be a general absorption in the green and red end of the spectrum. This, however, is not so, as there was ample visual evidence of the presence of a continuous group of lines in this region. The exposure of the arc was much shorter in this instance, and this may account for the inability of the plate to pick out the missing lines. The general absorption, confined to the violet end of the spectrum, was characteristic of the human fat pigments examined.

In the second spectrophotograph (Fig. 2, Plate I) the first picture is that of the copper arc spectrum extending from $\lambda = 5770$ to the ultra violet;

the second is of pig fat pigment; the third is the absorption spectrum of snake fat dissolved in ether; the fourth is that of tomato pigment (lycopin) in alcohol, and the last is that of fat pigment removed from a case of fibro-sarcoma.

A brief examination of this plate will show that absorption is least with snake fat pigment. It is harder than human fat, being of the same degree of consistency as mutton fat, and, in common with the latter, follows the general rule that saturation increase of the containing fat leads to a decrease in the intensity of pigmentation. Its fat pigment gives an absorption band between $\lambda = 5050$ and $\lambda = 4860$, and from $\lambda = 4050$ there is general absorption.

The adipochrome from pig fat, which gave a greenish tinge on straining, shows a distinct absorption band between $\lambda = 4860$ and $\lambda = 5050$; also between $\lambda = 4300$ and $\lambda = 4050$. Beyond $\lambda = 4050$ there is general absorption. There is also a suggestion of absorption between $\lambda = 4300$ and $\lambda = 4400$. Chlorophyll in living nettle leaf gives a corresponding absorption between $\lambda = 4300$ and $\lambda = 4510$. The absorption of pig fat pigment is very similar to that of urobilin in chloroform-alcoholic solution. With this solvent, urobilin exhibits one band between $\lambda = 5050$ and $\lambda = 4860$ with violet and ultra-violet absorption commencing at $\lambda = 4300$. Carotene in alcohol gives two bands; the first lies between $\lambda = 5000$ and $\lambda = 4810$ and the second between $\lambda = 4650$ and $\lambda = 4500$. From $\lambda = 4290$ there is general absorption. Xanthophyll in the same solvent shows absorption between $\lambda = 4550$ and $\lambda = 4450$. General absorption commences at $\lambda = 4150$. With lycopin there is a diffuse absorption in the blue and violet with general absorption in the ultra-violet; and in the last photograph—the mammary fat pigment from a case of fibro-sarcoma (solvent, ether)—there is an absorption between $\lambda = 4860$ and $\lambda = 5050$. Beyond $\lambda = 4500$ there is general absorption.

A careful analysis of the above absorption ranges and an examination of the plate will demonstrate that pathological human fat pigment, as also several cases of fat pigment from other animal sources, presents few absorption features in common with carotene, xanthophyll, or fucoxanthin. One case is cited above of absorption in the visible region of fat pigment isolated from pathological human fat, but this phenomenon is rather the exception than the rule. In this laboratory, many pathological fats and several normal fats have been examined during the course of the year by the direct vision spectroscope and the human fat pigment above referred to stood alone in giving selective absorption, which was found to correspond closely to that of urobilin. Otherwise, the absorption was of the general nature shown in Fig. 1, Plate I. This type of absorption emphasises the diactinic nature of lipochrome, and, in this respect, the latter corresponds closely with the unsaturated aliphatic hydrocarbons.

A formula for adipochrome.

With adipochrome isolated from fat invaded by carcinoma a combustion was carried out. The quantity of material used was very small, 0·005 g. The results of the combustion are given below:

0·005 g. adipochrome gave 0·00580 g. H_2O; 0·00064 g. H
" " 0·016 g. CO_2; 0·00436 g. C

which gives a formula of $C_{36}H_{64}$ for the pigment.

Carotene and its isomeride, lycopin, obtained from tomato, have the formula $C_{40}H_{56}$.

Discussion of Results.

The foregoing data, though by no means of an exhaustive nature, help to throw some light on the constitution of the pigment of fatty tissues.

It was thought that adipochrome might stand in relation to haemoglobin in much the same manner as anthocyanin and carotene to chlorophyll. This postulates pigmentary endogenesis, and in face of our evidence, we see no valid reason for supposing that the pigment equilibrium is controlled by external influences of changing and uncertain value, and that, in common with adipose tissue fat, its response to specific ingested substances (exochromes)[1] is immediate and involves structural modification of the pigment molecule.

Increase of pigmentation runs a parallel course with increase of unsaturation of adjacent fat, and the data at our disposal point strongly to the fact that this increase of pigmentation is expressive of increased manufacture of adipochrome *from fat alone.*

We have seen that adipochrome simulates unsaturated fatty acid in its behaviour with osmic acid and with bromine water. It differs from fatty acid in its behaviour with anhydrous arsenic chloride.

Anthocyanin, on the other hand, contains a substituted benzene ring (phenolic substitution) coupled up with a sugar group; adipochrome has been shown here to contain no aromatic group. Further points of divergence in properties from anthocyanin are demonstrated by the inertness of fat pigment in presence of sulphur dioxide, ferric chloride and sodium bisulphite, the ease with which it goes into solution in the common fat solvents and the difference in its absorption spectrum.

It is too often assumed that spectroscopic evidence alone is sufficient to establish the identity of a chemical compound. It is useful in distinguishing between desmotropic or isodynamically isomeric substances, but in our instance, we do not know if adipochrome exhibits desmotropism. It is quite unlikely that it does because of its paraffinoid nature. Even were it distinctly merolabile, we know nothing of its molecular configuration and a very small

[1] In this laboratory we have fed several mice exclusively on carrots over a period of six weeks, and on examination of the intestinal contents after death, abundant evidence, both spectroscopic and chemical, was forthcoming of the presence of carotene. No trace of this substance was obtained in the depôt fat of the mouse.

concentration of isomers would, if present, offer an insuperable obstacle to spectroscopic differentiation. We have to be content with taking the spectroscopic evidence, with the melting point, for instance, as a criterion of purity and as an index of a certain structural individuality. Beyond that we cannot go; the remaining chemical evidence tends to show that the adipochrome is of a paraffinoid nature and very closely related in structure to the fat with which it is associated.

Biological Significance.

It cannot be said that animal fat pigments are so amenable to classification as are plant pigments like anthocyanins, and the lack of coherent data for the correlation of their function and chemical structure impedes progress in marked degree in the ascertainment of their biological significance. In the body, pathological change manifests itself in many diverse pigmentary ways, *e.g.* in the red degeneration of submucous fibroids of the uterus, in the increased pigmentation of fat, bone marrow, and melanotic growths, in myeloid degeneration, in the ochronosis of cartilage, in chloroma, in hemochromatosis and in icterus.

In the already pigmented areas, increase in pigmentation may be due in some cases to an increase in concentration of the pigmenting substance; in other cases it may be due to the accession of a new structural entity; in others again there may be a working combination of both factors to produce a certain pathological end.

An example of the first condition is given by fat pigment local to a carcinomatous or sarcomatous lesion. In the second instance we may refer to the colour change produced in the skin after a heavy X-ray dosage. The mechanical effect of X-ray bombardment, coupled with X-ray absorption is the chief stimulus contributory to melanin change. The pigment accession at the site of a bruise is analogous to the pigmentation produced in plants by an unnatural accumulation of synthetic products, by trauma, by the puncture of insects in the plant leaf or stem, by fungal attack, or by blocking of the translocation current.

We can, however, hardly regard a tumour mass as a traumatic or a blocking factor in the modification of the comparatively inert adipochrome, for there seems to be no visible interference with the ordinary course of fat supply and distribution. It would seem that the increased pigmentation is partly due to bacterial attack intracarcinomatously, the associated pigment formed being transported to the neighbouring seat of storage. Thus, increased pigmentation in fat near to a malignant growth is of epiphenomenal rather than of prophenomenal import, and we can say that the manifestations of hyperchromatism or of chromidiosis in the tumour cell produce but a negligible response in the distant fat areas.

Adipochrome as an incitant, or cell proliferant, must, therefore, be ruled out of the category of such substances as Sharlach R (azo-*o*-tolueneazo-β-

naphthol) which produces an abundant epithelial proliferation in which both normal somatic and reduced mitoses occur [Barratt, 1907] and its gradual accumulation in its fat solvent might be regarded as a compensatory provision for the exothermic changes which the nutrient fat undergoes in response to the increased demand of the rapidly proliferating cancer cell.

SUMMARY.

1. Adipochrome is an unsaturated aliphatic hydrocarbon.

2. It is not related to carotene, xanthophyll, fucoxanthin, the anthocyanins, or the melanins.

3. It would seem to be manufactured from associated fat.

4. An account of its properties is given. *In vitro* it is comparatively inert chemically.

5. It is not a direct cell proliferant but may aid proliferation indirectly in virtue of its structural relationship to fatty acid.

6. Adipochrome of the pathological cases worked on (fats invaded by carcinoma, sarcoma) is not different from adipochrome in normal fat.

7. In cancer, there is an increase in adipochrome concentration in fat local to the growth.

8. The increased pigmentation is not due to chromoclasis but to an accessory chromosynthetic activity.

9. In the causation of cancer adipochrome is of epiphenomenal rather than of prophenomenal import, and bears no relationship to the chromidiosis of the tumour cell.

In conclusion, the writer desires to express his best thanks to Prof. Robert Muir, F.R.S., Prof. D. Noël Paton, F.R.S. and Sir George T. Beatson, for the kindly interest they have shown and the encouragement they have given him in the prosecution of the research. His best thanks are also due to the Medical Research Council for their provision of a personal grant.

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DESCRIPTION OF PLATE.

Fig. 1. Fat pigment from a case of fibro-adenoma. No selective absorption in evidence; general absorption in violet.

Fig. 2. The first spectrophotograph is that of the copper arc; the second, that of pig fat pigment dissolved in ether; the third, that of snake fat, also dissolved in ether; the fourth, that of lycopin dissolved in alcohol; the last is that of fat pigment (mammary origin) from a case of fibro-sarcoma. Solvent, butyl alcohol.

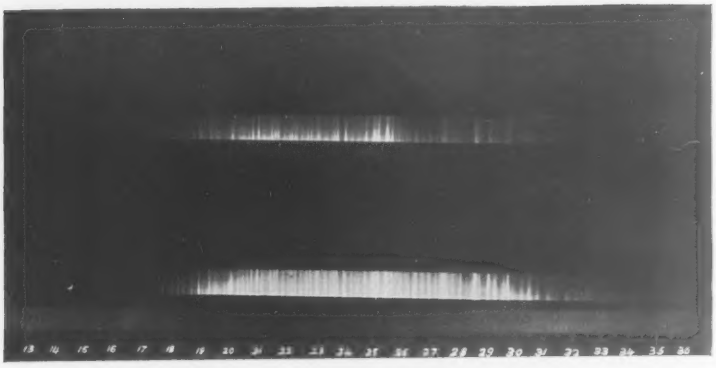

Fig. 1.

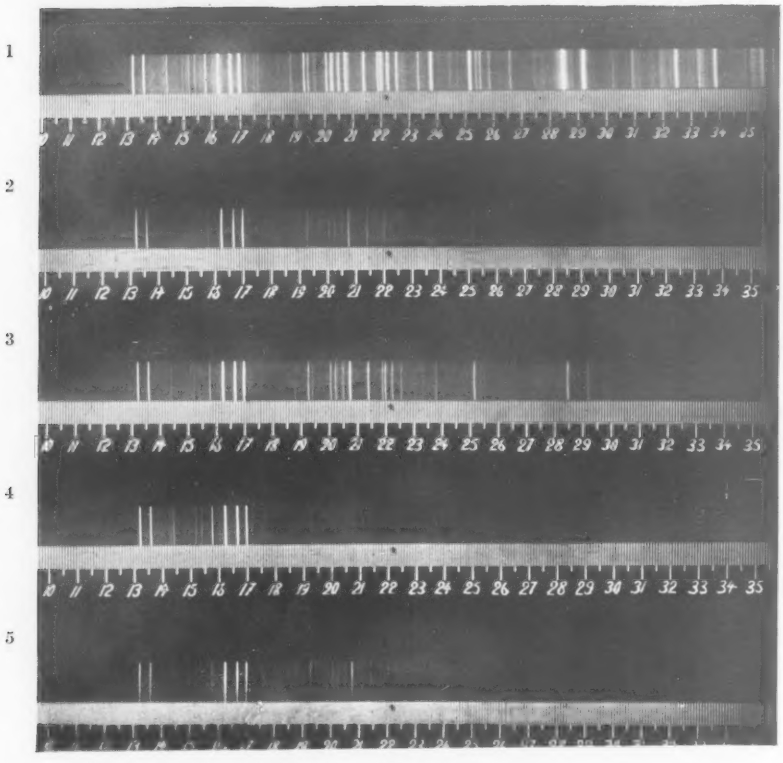

Fig. 2.

XXXIV. THE ACTION OF RENNET AND OF HEAT ON MILK.

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(*Received November 5th, 1923.*)

I. THE ACTION OF RENNET ON MILK.

Introduction.

The action of rennet on milk has been the subject of a number of investigations. Hammarsten [1872] was the first to show that the coagulation of milk by rennet was due to a soluble enzyme which, he supposed, acted on the caseinogen to produce an insoluble curd (Käse or Casein) and a soluble whey-protein (Molkeneiweiss).

Bosworth [1913] showed that the production of the latter was not due to the rennet action, and that the insoluble casein was the sole product of the reaction, a result confirmed by Harden and Macallum [1915]. Bosworth further confirmed the fact that the actual coagulation was not due to the rennet, but was a secondary effect due to the difference in solubility of the salts of casein.

Bosworth and Van Slyke [1913] in the course of extended investigations demonstrated that caseinogen and casein were analytically indistinguishable, a result also obtained by Geake [1914]. The former investigators claimed however that the salts of casein contained twice the amount of basic elements present in those of caseinogen, and on the basis of their analytical results they calculated the molecular weight of caseinogen to be 8888, and that of casein 4444. The general conclusion at which they arrived was that the rennet caused a proteolytic cleavage of the caseinogen molecule into two molecules of casein, which could then be coagulated by the addition of bivalent metal ions.

Other theories of rennet action have been put forward. Thus J. Mellanby [1912] supposed that the enzyme was adsorbed by the caseinogen and that it was the precipitation of the enzyme-caseinogen complex which caused the clot in milk. Alexander [1912] suggested that under normal conditions the caseinogen was protected by the lactalbumin of the milk, and that the enzyme, by destroying the lactalbumin, permitted the coalescence of the caseinogen particles. Schryver [1913] advanced a similar theory in which the coagulation was held to be due to the surface tension effects on the colloid constituents of the milk.

Up to the present time the only definite evidence of difference between caseinogen and casein is that based on the work of Bosworth and Van Slyke quoted above. The present investigation was commenced with the object of attempting to differentiate the two proteins by means of a study of their relative optical rotation and racemisation curves.

Experimental.

Woodman [1921] has shown that the identity of proteins may be determined with great accuracy by studying the curve of racemisation, namely the rate of racemisation of the protein in strong alkali as judged by the progressive diminution in optical rotation during incubation at 37°. Such a method is sufficiently sensitive to differentiate proteins as similar as serum albumin and lactalbumin, where other methods, biological and chemical, have failed to do so.

If the action of rennet is to cause a proteolytic cleavage of the caseinogen molecule, as suggested by Bosworth and van Slyke, we should expect to obtain different initial values of optical rotation, and possibly different rates of racemisation with caseinogen and its cleavage product. If on the other hand the action of rennet in producing a clot is simply a colloidal phenomenon, as suggested by other workers, then we should expect the racemisation curves of caseinogen and casein to be identical.

For the preparation of the proteins the method of Bosworth and Van Slyke was used, with the exception that 0·2 % NaOH was substituted for NH_4OH, in order to avoid possible high nitrogen values due to contamination with traces of ammonium salts. In order to determine whether alkali of this concentration effected any racemisation, a control preparation was made by the method of Baker and Van Slyke [1918], in which contact with alkali was avoided. The results (shown below) indicate that the dilute alkali does not cause appreciable racemisation.

Equal quantities of the caseinogen and casein were dissolved in alkali under standard conditions, the solutions incubated at 37°, and the optical rotations observed at intervals. The concentration of protein was checked by nitrogen determinations, and the specific rotation calculated. The readings of the first 24 hours were rendered difficult by the gradual formation of a slight suspension in the solution, which was found to consist of inorganic phosphate split off from the caseinogen by the action of alkali [Plimmer and Scott, 1908]. This suspension was completely precipitated by the end of the first day, and could be filtered off to give a clear solution, while the errors caused by the temporary turbidity were quite insufficient to invalidate any of the readings obtained.

Determinations were carried out on duplicate solutions and with several different preparations of caseinogen and casein and in all cases good agreement was obtained. The following is a summary of the results:

Table I. *Specific rotations* (2 % *in N/2 NaOH*).

Hours	*Caseinogen* (Baker and Van Slyke preparation)	*Caseinogen* (Bosworth and Van Slyke preparation)	*Casein* (Bosworth and Van Slyke preparation)
½	−103·0	−102·5	−103·5
2½	—	− 89·5	− 88·5
6	− 80·0	− 79·0	− 79·0
9	—	− 74·5	− 74·5
24	− 66·0	− 64·5	− 64·5
50	− 55·0	− 55·0	− 55·0
100	− 42·5	− 42·5	− 42·0
169	− 38·0	− 39·0	− 38·0

Discussion of Results.

It would appear from the results above that the two proteins, caseinogen and casein, must be considered chemically identical, a view which is in harmony with the fact that exhaustive analyses by several workers have shown them to be analytically indistinguishable.

The evidence against such a view is two-fold: (*a*) the difference in solubility of caseinogen after treatment with rennet, and (*b*) the difference in combining capacities of the two proteins. The first objection may be met by assuming that the rennet causes a change in the colloid condition of the caseinogen which facilitates precipitation in the presence of bivalent metal ions. With regard to the second objection, it seems doubtful whether the method used is in itself capable of sufficient accuracy to form a basis of differentiation, and in choosing between the conflicting evidence, one would rely rather on a physical method as being capable of the greater precision.

It has been urged in favour of the "cleavage" theory that the function of rennet is to cause the preliminary splitting of the large caseinogen molecule prior to more complete digestion by the ordinary proteolytic enzymes. Such a view may be met by the equally probable explanation that rennet coagulates the caseinogen of milk in order to allow the protein to be retained for a longer period in the sphere of action of the digestive juices.

II. THE EFFECT OF HEAT ON MILK.

Introduction.

The effect of heat on milk is here studied only in relation to its action on the caseinogen, a subject which has received comparatively little investigation.

Jensen and Plattner [1904] observed that the heating of milk to high temperatures caused the development of a brown coloration, and they supposed this to be due to the incipient breakdown of the caseinogen molecule. A number of investigators have observed that autoclaving milk at high temperatures (150°) causes actual coagulation of the caseinogen. Recently Hammarsten [1918] has claimed from his investigations to have shown that heating at 116° in an autoclave causes the splitting-off of an albumose-like body from the caseinogen.

From the nutritional side the subject has received more attention. Earlier work was directed to a study of the comparative digestibility of the raw and heated product. For a full discussion of the subject reference may be made to Lane-Claypon [1916] but it may be said that the general opinion tends to show that heated caseinogen is, on the whole, *more* digestible than unheated caseinogen. McCollum and Davis [1915], however, in experiments in which they heated caseinogen in an autoclave at 15 lbs. pressure, brought forward evidence that the heated product was no longer adequate to support growth, and claimed that "heat destroyed its biological value as a complete protein." Contrary evidence is forthcoming from Funk and Macallum [1916] and from Hogan [1917] both of whom base the loss of nutritive value on destruction of vitamins rather than alteration in the caseinogen molecule itself. Their opinion is supported by Hammarsten [1918] who concluded from his experiments that "inability of heated caseinogen to maintain growth of young rats cannot be ascribed to altered structure of constituent amino-acids."

The whole question is however in an unsatisfactory state, and it seemed that further investigation, particularly from the chemical standpoint was necessary.

Woodman's method of following the racemisation curve of a protein as a means of identification seems to offer excellent opportunity of detecting qualitatively whether any fundamental change takes place in the caseinogen molecule on the application of heat. If there is any alteration in, or displacement of, the amino-acid content, we should expect such a method to give a most sensitive indication of the change.

Experimental.

Fresh whole milk was taken and the cream removed by centrifuging. Equal quantities (1 litre) were treated separately as follows:

(*a*) Unheated (control).

(*b*) Pasteurised at 60° for half an hour.

(*c*) Boiled under reflux condenser for half an hour.

(*d*) Autoclaved at 120° for half an hour.

The various solutions were then precipitated with acetic acid and the caseinogen separated and purified by the method of Bosworth and Van Slyke. All the products were obtained finally as fine powders, (*a*) and (*b*) white, (*c*) slightly yellowish, and (*d*) light sand-coloured. The two former gave colourless solutions in NaOH, (*c*) slightly yellowish, but (*d*) gave a distinct reddish-brown solution which in high concentrations had a tendency to become gelatinous. The coloration could not be removed by water or any of the common solvents, or by dialysis.

Equal quantities of these four preparations were dissolved in standard alkali and their racemisation curves observed as in the previous experiments (see p. 246). The following is a summary of the results obtained:

Table II. *Effect of heat on caseinogen.*

Hours	Specific rotations (2 % in $N/2$ NaOH) from raw milk	from pasteurised milk	from boiled milk	from autoclaved milk
2½	−89·5	−88·0	−92·0	−91·0
9	−74·5	−76·0	−74·0	—
24	−64·5	−65·0	−67·0	−67·5
50	−55·0	−55·0	−52·0	−56·5
100	−42·5	−44·0	−43·5	−42·0
169	−39·0	−38·0	−37·0	−37·0

Discussion.

These results show clearly that, within the limits of the temperatures studied, heat effects no alteration in the constitution of the caseinogen molecule.

There remains to be explained the coloration and susceptibility to gelatinisation of the autoclaved product, and the increased digestibility as found by Hammarsten and other workers. The results obtained above indicate that alteration in physical state can be the only cause of the two latter phenomena. This fact is borne out strikingly by the experiments of Brenneman [1913] on the cause of increased digestibility. By using as a subject for his experiments a boy who could without discomfort regurgitate his food, he found that whereas with raw milk, the curds formed in the stomach were large and tough, with boiled milk they were soft and finer in character. Such an alteration in texture is explained on the basis of a difference in colloid condition of the raw and boiled caseinogen.

With regard to coloration, a control experiment was carried out in order to determine whether the caseinogen itself was the origin of the browning in autoclaved milk. A neutral solution of 4 % calcium caseinogenate (made by dissolving caseinogen in excess of $CaCO_3$ and filtering off excess of carbonate) was autoclaved for half an hour at 120°. There was no change in colour and the racemisation curve corresponded to those previously obtained.

Caseinogen from autoclaved calcium caseinogenate, 2 % in N/2 NaOH.

Hours	Specific rotation
2½	−90·0
9	−75·0
24	−65·0
50	−56·0
100	−43·0
169	−38·5

It follows therefore that the coloration obtained by autoclaving milk is due to the action of heat on some constituent other than the caseinogen, and that the apparently permanent coloration of caseinogen precipitated from such milk is due to the adsorption of pigment by the protein, and not to alteration in the caseinogen molecule itself.

The cause of coloration in autoclaved milk.

In order to determine which constituent of milk is responsible for the coloration of the autoclaved product, solutions of the following percentage composition were made up and autoclaved at 120°.

(*a*) Calcium caseinogenate, 4 %.
(*b*) Calcium caseinogenate, 4 %.
Lactose, 4 %.
(*c*) Calcium caseinogenate, 4 %.
Cream, 5 %.
(*d*) Lactose, 4 %.

At the end of half an hour, (*a*) remained white, (*b*) was dark brown, (*c*) slightly tinted, and (*d*) light yellowish brown. These experiments indicate that lactose is the precursor of the pigment in autoclaved milk. The slight coloration in (*c*) was probably due to traces of lactose in the cream, while the marked difference between (*b*) and (*d*) may show that the reaction is catalysed in the presence of a colloid substrate of calcium caseinogenate.

On acidifying (*b*), a precipitate was thrown down which adsorbed the pigment, giving on purification a light sand-coloured powder identical with that obtained from autoclaved milk.

Summary.

(1) The action of rennet and of heat on solutions of caseinogen has been investigated by means of a study of the racemisation curves of the raw and treated protein.

(2) It is shown that caseinogen and casein have identical racemisation curves, evidence which supports the view that rennet does not cause any proteolytic cleavage of the caseinogen molecule but that coagulation is due to an alteration in the colloid state of the caseinogen by which precipitation in the presence of bivalent metal ions is facilitated.

(3) Within the limits of the temperature studied (up to 120° for half an hour) heat does not affect the constitution of the caseinogen molecule. Any difference in digestibility must be due to alteration in the physical (colloid) condition of the protein.

(4) The coloration of autoclaved milk is due to the caramelisation of the lactose, a reaction which appears to be catalysed by the presence of a colloid substrate of calcium caseinogenate.

The colour of caseinogen precipitated from autoclaved milk is due to adsorption of this pigment, and not to alteration in the caseinogen molecule itself.

In conclusion I should like to express my gratitude to Dr H. E. Woodman for his constant help and advice, and to Prof. T. B. Wood, F.R.S., for the facilities which he has kindly placed at my disposal for carrying out this investigation.

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XXXV. A METHOD FOR THE ESTIMATION OF UREA IN 0·1 c.c. OF BLOOD.

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(*Received November 29th*, 1923.)

In connection with some investigations upon which we were engaged it was necessary to make frequent estimations of the urea content of the blood in man. The methods at present in use for the estimation of blood urea, such as that of Folin and Wu, require several cc. of blood which must be obtained by venipuncture. When blood is required at frequent intervals venipuncture is not always advisable and it was necessary to devise a method which would be suitable for such amounts of blood as could be readily obtained by pricking a finger.

The method described below is simple, accurate and requires only 0·1 cc. of blood for each estimation. We always take two samples of blood in order to obtain duplicate figures.

A 6″ by 1″ Jena test-tube is fitted with a rubber stopper to which are attached two tubes. One drawn out to a large capillary bore reaches almost but not quite to the bottom of the test-tube. The other is bent twice at right angles and is drawn out at its free end into a large capillary tube. This second capillary tube dips into a long narrow 5 cc. cylinder, reaching nearly to the bottom. The blood is collected, after pricking the finger, in a long capillary pipette which has been carefully calibrated by mercury to contain 0·1 cc. It is immediately placed at the bottom of the test-tube and the pipette washed out with two lots of 0·1 cc. of urease solution prepared according to the method of Folin and Wu [1919]. One drop of phosphate solution (Folin and Wu) is also added. The urease solution can be prepared either from Jack bean or a high grade Soy bean. The tube is then put in a rack in a water-bath kept at 54° and allowed to remain there for 5–10 minutes in order to decompose the urea. In the graduated cylinder are placed 2 cc. of $N/20$ sulphuric acid.

To the test-tube containing the blood, upon which the urease has acted, are then added five drops of a saturated solution of borax and the apparatus connected up, the test-tube remaining in the water-bath at 54°.

A current of air, driven by a blower and dried by bubbling through concentrated sulphuric acid, is blown through the tube. The air does not bubble through the blood and borax solution but impinges on its surface, so that there is no trouble from frothing, and then bubbles through the $N/20$ sulphuric acid. The current of air should be slow for the first few minutes and thereafter as rapid as possible and is continued until the contents of the test-tube are dry. This takes about half an hour. With the air current still running the cylinder is then lowered from the capillary tube and this tube washed down.

To the contents of the cylinder is then added 1 cc. of Nessler solution (Folin and Wu) and the whole made up to 5 cc. with water. A standard is prepared in a similar cylinder containing 2 cc. of $N/20$ H_2SO_4, 1 cc. of ammonium sulphate solution containing 1 mgm. N per 100 cc., 1 cc. of Nessler solution and 1 cc. of water. The unknown and standard are then compared in a small Duboscq colorimeter with the standard set at 20.

$$\frac{20}{\text{reading of unknown}} \times 10 = \text{mg. urea N per 100 cc. blood.}$$

The ammonium sulphate solution is made up from a stronger solution and will keep for at least a week. The rubber stoppers and rubber tubing used in the apparatus should be prepared by soaking first in sodium hydroxide solution to remove free sulphur etc. and then boiled in dilute sulphuric acid and frequent changes of distilled water, otherwise anomalous results may be obtained. The accuracy of the method was tested in several ways.

A urea solution was made up containing 15 mg. N per 100 cc. The results obtained with this solution in place of blood were: 14·8, 14·85, 15·0, 15·4, 15·0, 15·3, 15·2, 14·9.

A goat's blood gave 14·08 mg. urea N per 100 cc. by the method of Folin and Wu and in duplicates by the new method 14·1 and 14·05. A concentrated solution of urea was added to rabbit's blood and to ox blood.

	mg. urea N
Rabbit's blood...	18·6, 18·4
„ „ +13·8 mg. urea N per 100 cc.	32·4, 32·2
Urea recovered	13·8
Ox blood	7·5
„ „ +13·8 mg. urea N per 100 cc. ...	21·2
Urea recovered	13·7

Unselected duplicate estimations on a series of normal young men and women:

Blood urea N mg.		Blood urea N mg.		Blood urea N mg.	
13·4	13·4	9·7	9·4	10·9	10·8
10·5	10·3	11·9	12·1	9·9	9·9
14·2	14·1	8·7	8·7	9·2	9·2
11·6	11·7	10·4	10·6	10·9	11·0
11·5	11·7	15·9	16·1	11·1	11·1
10·5	10·5	16·8	16·8	11·4	11·8
8·9	8·8	14·4	14·5		

As an example of the application of the method the following experiment may be quoted. The subject fasted from the previous meal until 9.30 a.m. when 50 g. of protein in the form of beef steak was eaten.

	Blood urea N	Urine urea
	mg.	g.
10.15 a.m.	13·7	1·11
11.15 „	14·7	1·22
12.15 p.m.	12·6	1·06
1.15 „	14·8	1·37
2.15 „	18·3	1·70
3.15 „	15·6	1·58
4.15 „	15·4	1·55
5.15 „	15·5	1.40

REFERENCE.

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XXXVI. HAEMOGLOBIN AND METHAEMOGLOBIN AS OXIDATIVE CATALYSTS.

By MURIEL ELAINE ROBINSON.

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(*Received December 11th, 1923.*)

THAT iron plays a part as a catalyst in the living cell is inherently probable. The work of Warburg and Meyerhof, upon sea urchin eggs, has provided experimental evidence to show that it catalyses biological oxidations, and Warburg [1914] has claimed for it a predominant influence in respiratory processes. For the most part, experimental studies bearing on this matter have dealt with the effect of adding inorganic ionisable iron salts to preparations from living tissues, or to tissue constituents such as lecithin [Thunberg, 1910]. The tissues, however, contain organic compounds of iron, and among these are haemoglobin and, probably, some of its derivatives.

So far back as 1865 Kühne showed definitely that haemoglobin is present in blood-free muscle fibres. He then suggested that the pigment played some part in the oxidation processes of the contractile substance, his view being supported by the familiar fact that red muscles, which contain most of the pigments are those most capable of sustained activity.

No attempt seems to have been made to test this suggestion directly. It is well known, of course, that haemoglobin and those of its derivatives which contain iron can act as peroxidases or pseudoperoxidases [Buckmaster, 1907] in the sense that they promote the blueing of guaiacum tincture by peroxides, but this fact brings no proof that they play a part in oxidations which have metabolic importance.

At Prof. Hopkins' suggestion the following experiments were therefore undertaken. They deal especially with haemoglobin, methaemoglobin and haemin as catalysts in the oxidation of unsaturated fats.

MacMunn [1884, 1887], as is well known, claimed that in some cases at least, the pigments present in the tissues are special derivatives of blood pigments which, in the case of muscle, he called myohaematins. Whether his view be correct would seem still to be an open question. No attempt has yet been made to separate these substances in a form in which their catalytic activity could be tested.

The substrate actually employed in my observations was linseed oil. This is, of course, a drying oil, and clearly the results obtained might be supposed

to come into close relation with the whole subject of "driers," which are known to act as catalysts of oxidation of oils. This subject has been studied on scientific lines by E. Rideal and others. Rideal and Taylor [1919] point out that efficient driers are usually oxides or salts of metals which can exist in two states of oxidation; they act most effectively when added in some form which is soluble in the oil, cobalt, for instance, in the form of a linoleate, manganese as rosinate, etc.

The curves of oxygen uptake given by Rideal indicate that the uncatalysed autoxidation shows the typical sinuous curve of autocatalysis, while in the catalysed action the period of induction still exists, though it is reduced. It is therefore postulated that the catalytic action depends on an increased production of an autocatalytic agent, normally present in small quantities, which is probably of peroxide form, analogous to that formed by turpentine. The autocatalyst originally present is said to be destroyed by boiling.

The subject has also been studied by Coffey [1922] by the method of measuring the increase of weight of oil spread in very thin films on a glass plate, and by a manometric method, when pieces of filter paper were soaked in a solution of oil in light petroleum and the gaseous exchanges were studied. In the latter case, evidence of CO_2 formation among other volatile decomposition products was obtained. A period of induction of about 15 minutes was a feature of these results.

The apparatus used in the present research was the Barcroft differential manometer, with a bottle of suitably modified form which enables oxidation to be effected more rapidly than is the case for the original pyriform shape, owing to larger surfaces exposed to the air or other gas filling the bottle, which is calibrated for 3 cc. of material. A cup within the bottle may contain 40 % potash so that any carbon dioxide evolved may be absorbed and approximately estimated.

The linseed oil was used in the form of an emulsion, in water or buffer solution, 50 mg. of oil being shaken up with 10 cc. of water or buffer, and 1 or 2 cc. of the ensuing uniform suspension being used for each experiment.

As the oxidation of the oil in my experiments was carried out in a system physically different from that existing in the investigations of the authors just quoted, it seemed well to study the effects of known "driers" when added to an emulsion and shaken in the Barcroft apparatus. The experiments throughout were carried out at 37°. Experiments with cobalt oxide gave a curve for oxygen uptake showing the typical sinuous form obtained by Rideal and others, with a well marked preliminary induction period of about an hour. In control experiments, that is in absence of the catalyst, the oxygen uptake was exceedingly small and never attained appreciable dimensions in nine hours (Fig. 1).

Before describing in detail my observations on blood pigments, it may be stated generally that haemoglobin, methaemoglobin and haemin catalyse with efficiency the autoxidation of linseed oil, in which linolenic acid is doubtless

the constituent chiefly responsible for oxygen uptake. Haematoporphyrin, on the other hand, is without effect. While it would seem, therefore, that the presence of iron in the molecule is essential to the catalytic activity, it is much more difficult to decide whether the mechanism of catalysis is identical with that of inorganic driers in general.

The samples of haemoglobin used were supplied by Mr T. R. Parsons and were prepared from sheep's blood by the freezing centrifuge method. The samples were therefore free from reagents, except possible traces of sodium chloride.

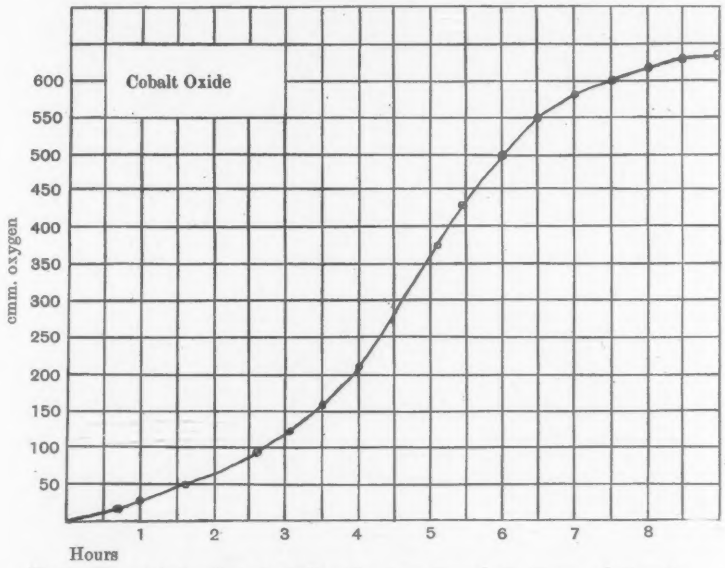

Fig. 1. The catalytic effect of cobalt oxide. 5 mg. linseed oil. 50 mg. cobalt oxide.

Using 1 cc. of a haemoglobin solution, containing 1 mg. haemoglobin per cc. a marked catalysis of oxidation is found to take place, differing from that of the so-called driers in that the preliminary induction period is absent. The acceleration of oxygen uptake, while more or less proportionate to the amount of haemoglobin present when the latter is at low concentrations, failed to increase proportionately for higher concentrations (Fig. 2). Corresponding methaemoglobin solutions, made from crystalline methaemoglobin prepared in a similar manner to the haemoglobin used, were found to exhibit similar catalytic activity, as shown in Fig. 3, where the effects of solutions of haemoglobin and methaemoglobin of equal concentrations are compared. The slight differences in the shapes of the curves for haemoglobin obtained from day to day are due to slightly altered rates of shaking caused by unavoidable variations of current.

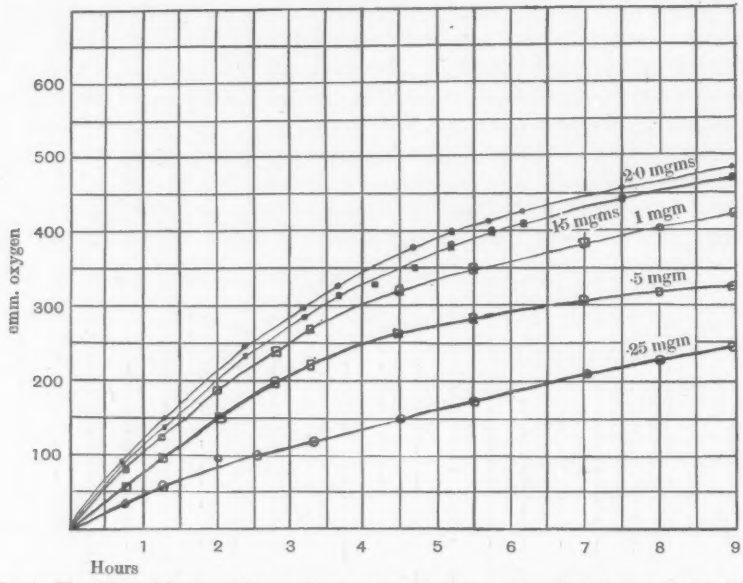

Fig. 2. The effect of haemoglobin, in different concentrations. 5 mg. linseed oil with 2·0, 1·5, 1, ·5 and ·25 mg. of haemoglobin. Control curve (oil without catalyst) as in Fig. 3.

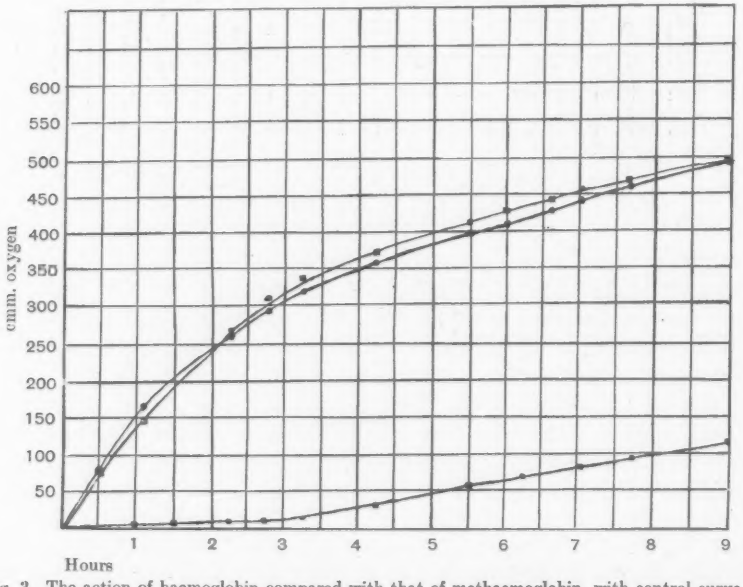

Fig. 3. The action of haemoglobin compared with that of methaemoglobin, with control curve 5 mg. linseed oil with 5 mg. haemoglobin, and the same with 5 mg. methaemoglobin.

There would seem to be little doubt that the oxygen in oxyhaemoglobin is in some special relation with the iron. Apart from general probabilities the work of Laidlaw [1904], showing that the entry of oxygen into the haemoglobin molecule stabilises the iron, offers significant evidence for this. If the pigment be an oxidative catalyst as well as (in so pre-eminent a degree) an oxygen carrier, and both in virtue of its iron content, its position in the former category might be expected to be exceptional. It would not seem, however, that the quite different association with oxygen, found in methaemoglobin, affects to any important degree, the catalytic effect of the molecule.

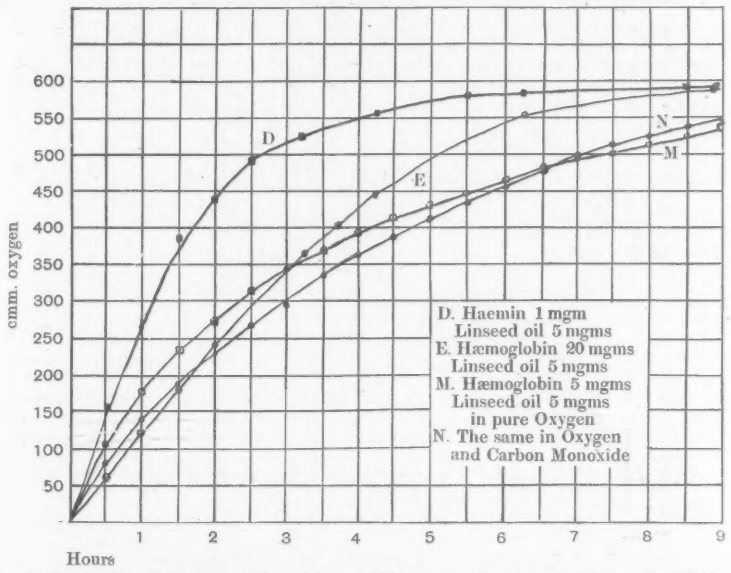

Fig. 4. *Curves N and M.* The action of haemoglobin in an atmosphere of one part of CO and five parts O_2 compared with that in pure oxygen. 5 mg. linseed oil. 5 mg. haemoglobin. *Curves D and E.* The action of haemin compared with that of a strong haemoglobin solution containing an approximately equivalent amount of iron. 5 mg. linseed oil with 1 mg. of haemin, and with 20 mg. of haemoglobin.

An attempt was then made to see if the seemingly catalytic activity of haemoglobin were removed by the action of carbon monoxide. The experiments were carried out in two ways:

(1) Coal gas, or pure carbon monoxide (prepared from formic acid) was bubbled for half an hour into the bottle of the Barcroft apparatus containing the haemoglobin to be used for the experiment. The linseed oil emulsion and buffer or water were then added, and the experiment carried out as usual.

(2) The oil emulsion and haemoglobin solution having been placed in the bottle, and the apparatus arranged for the experiment, the air in both bottles was replaced by a known gas mixture containing one part of carbon monoxide

and five parts of oxygen. In a control experiment carried out simultaneously, the bottles were filled with pure oxygen. No inhibition of catalysis, but rather a slight increase of activity was observed (Fig. 4, Curves *N* and *M*).

Haemin gave curves for catalysis of oxygen uptake of a type rather different from that observed for haemoglobin and methaemoglobin, the initial stages of oxidation being much more rapid (Fig. 4, Curves *D* and *E*). The concentration of iron in haemin is approximately twenty times greater than in haemoglobin. When very weak solutions of haemin were used, in which the iron content approximated to that in a haemoglobin solution containing 2 mg. haemoglobin per cc. the haemin curves tended to resemble those

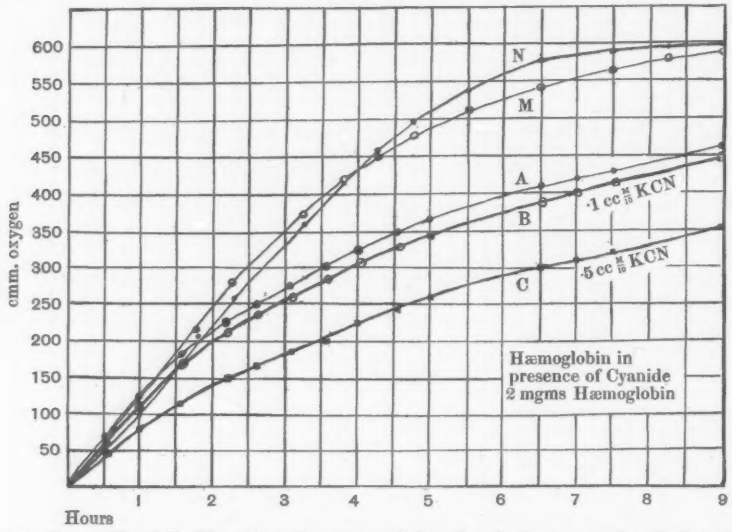

Fig. 5. *Curves M and N.* The action of a very weak haemin solution compared with that of a haemoglobin solution containing an approximately equivalent amount of iron. 5 mg. linseed oil with 0·2 mg. haemin, and with 4 mg. haemoglobin.
Curves A, B, C. The effect of cyanide in different concentrations. 5 mg. linseed oil and 2 mg. haemoglobin with 1 cc. and 0·5 cc. *M*/10 KCN.

obtained by the use of haemoglobin. The converse however, did not appear to be true, that is, increased concentrations of haemoglobin, such as solutions containing 20 mg. per cc. never gave rise to curves resembling the typical haemin curve in its initial stages (Fig. 5, Curves *M*, *N* and *A*; Fig. 4, Curves *D* and *E*). The difference may be in the colloidal character of haemoglobin.

As the results obtained seem to indicate action on the part of iron, particularly on account of the inactivity of haematoporphyrin, attempts were made to observe if the catalysis could be inhibited by the action of cyanide. Warburg has stated that, for the sea urchin's egg, the amount of cyanide necessary to cause total inhibition of respiration is that required to transform the iron present into the inactive ferrocyan-ion.

With haemoglobin and methaemoglobin, the addition of such a strength of cyanide was found to cause no change in the oxygen uptake; even the presence of $M/600$ potassium cyanide was practically without effect, this amount of cyanide being equivalent to five times the concentration of iron present. If, however, the strength of cyanide used were above $M/300$ a distinct but incomplete inhibition was observed (Fig. 5, Curves *A*, *B*, *C*). This effect however was found to be entirely absent in the case of haemin. These results are difficult to interpret; it is assumed that the cyanide exerts an effect on the colloidal catalysts owing perhaps to some change in the physical system, particularly as the inhibition is much more marked if a solution of blood be used instead of one of crystalline haemoglobin. The addition of cyanide to

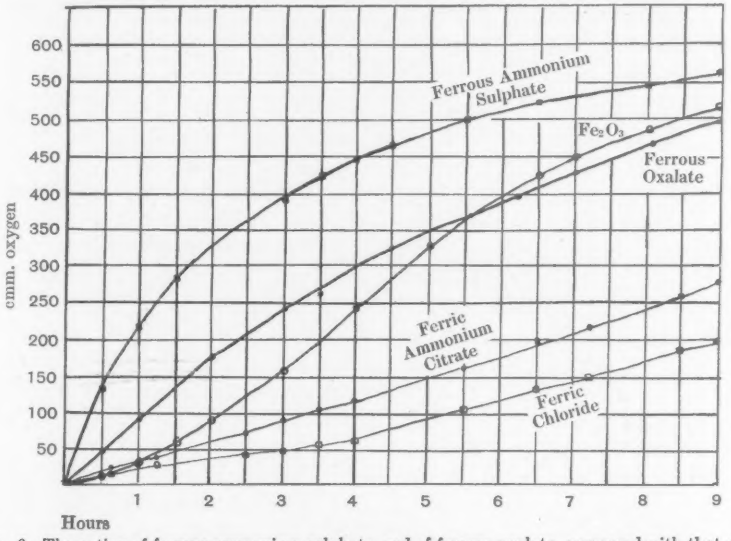

Fig. 6. The action of ferrous ammonium sulphate, and of ferrous oxalate, compared with that of iron sesquioxide and of ferric salts. 5 mg. linseed oil with 1 cc. ferrous ammonium sulphate (0·00112 g. iron); with 1 cc. saturated ferrous oxalate; with 50 mg. iron sesquioxide; and with solutions of ferric chloride and ferric ammonium citrate containing 0·00112 g. Fe in 1 cc.

haemoglobin and methaemoglobin brings about the formation of cyanohaemoglobin, as can be seen by the change in colour and in the spectrum. Apparently this pigment can also exert a catalytic effect.

It is noteworthy that the curves of oxygen uptake of linseed oil, when the blood pigments are employed as catalysts, show no signs of the period of induction which is so characteristic of those obtained in the presence of the driers referred to above. It would not seem, however, that this is universally present when the action of inorganic catalysts is concerned, indeed, to judge by my experiments, it is not seen in the case of such inorganic salts of iron as ferrous ammonium sulphate, ferrous oxalate and ferric ammonium citrate

(Fig. 6). Other ferrous salts have been difficult to obtain, except the lactate, which is very slightly soluble in cold water.

The ferric salts which were tried seemed to exhibit less catalytic activity. In this connection the work of Buckmaster may again be cited (Fig. 6). If an equivalent of cyanide be added to ferrous ammonium sulphate the resulting ferrocyanide exerts no catalytic effect.

It is noteworthy that if the concentration of the ferrous salt solution be such that the iron is present in similar strengths to those existing in the solutions of blood pigments, such as in haemoglobin solutions containing 1 mg. haemoglobin per cc. no catalysis is evident; the concentration of the solutions of ferrous salts used was such that 1 cc. contained 0·00112 g. iron, but the minimal amount that would exert catalytic activity was not determined.

Discussion.

The experiments described show conclusively that haemoglobin, methaemoglobin and haemin act in very low concentrations as efficient catalysts in the oxidation of linolenic acid. It is probable therefore that when blood pigments are found within the structural elements of a tissue they take a definite place among the agents which promote oxidation in that tissue. Since haematoporphyrin has no similar activity it seems clear (as might indeed be expected) that the above mentioned compounds function in virtue of their content of iron. In the case, at least, of the particular oxidation studied, haemoglobin and its derivatives are—if comparison be based upon the actual amount of iron present—more efficient as catalysts than inorganic salts of the metal. None of the accepted explanations applied to the influence of ionised iron as a catalyst seems however to fit the facts in the case of these organic compounds.

Since there is no doubt that the atom of iron in the haemoglobin molecule plays a primary part in oxygen carriage, it would appear likely that when haemoglobin acts as a catalyst of oxidations the same affinities of the iron atom would be concerned with the oxygen transfer there involved. It is remarkable therefore that methaemoglobin, in which the relation of oxygen to the iron atom is certainly different, and carbon monoxide haemoglobin, in which the locus of oxygen uptake is (presumably) occupied, should both, when in equal concentrations, act with a catalytic effect equal to that of haemoglobin itself. Cyanides, when added in concentrations at all commensurate with the amount of iron concerned, have no effect upon catalysis by these substances. If the accepted explanation of the effect of cyanides in inhibiting oxidations catalysed by inorganic iron is correct—namely, that it is due to the formation of an inactive complex ion—the absence of any effect upon non-ionised iron in the organic molecule might be expected. The fact, however, makes it even less easy to decide precisely how the iron exercises its influence in the phenomena described.

In the slow autoxidation of unsaturated fatty acids it is commonly assumed that the first occurrence is peroxide formation at the unsaturated linkages. This circumstance has been supposed to explain the fact that the curves of oxygen uptake show the characteristic of an autocatalysed reaction; oxidation increasing in velocity as the concentration of active oxygen rises.

Haemoglobin and its iron-containing derivatives are known to act as peroxidases or pseudoperoxidases, and it is possible that, in the case of the unsaturated fatty acids, actual oxidation of the molecule, by the active oxygen which first appears at the unsaturated linkage, is promoted by the presence of the organic iron compounds. Under their influence oxidation may proceed with a much lower concentration of peroxide than is the case for the uncatalysed reaction. On this view it is interesting to observe that the period of induction, notable in the spontaneous reaction, and representing the gradual attainment of an efficient concentration of active oxygen, disappears when haemoglobin or its derivatives are present. The system then pictured would be analogous to plant oxidase systems, as described by M. W. Onslow [1920]. In these, peroxide formation occurs in relation with adjacent hydroxy-groups of catechol derivatives. Actual oxidation of the catechol molecule follows upon such preliminary formation of peroxide, this process being greatly accelerated by a peroxidase.

Unfortunately, the precise lines on which peroxidases exert their action are unknown, and it would seem that the presence of a metal in their constitution is not essential to their activity. In any case it is of course possible that haemoglobin, in promoting the oxidation of fats, acts along other lines. The main purpose of the experiments described in this paper is to show that, apart from its function in the blood, haemoglobin, which was shown long ago by Kühne to be present in tissues, may play its own part in promoting oxidations.

Summary.

1. The blood pigments haemoglobin, methaemoglobin and haemin, are shown to act as efficient catalysts of the autoxidation of linseed oil; haematoporphyrin, however, shows no similar activity. The catalysis is attributed to the iron content of the molecule.

2. The concentration of iron, present as blood pigment, necessary to produce the same catalytic effect, is very much smaller than that required if the iron be in the form of an inorganic salt.

3. Addition of neutralised potassium cyanide in amounts equivalent to the iron present is without effect on the catalytic action. It is suggested that the slightly inhibitory effect of high concentrations of cyanide may be due to some physical change in the colloidal system.

I wish to thank Prof. Hopkins for suggesting this work and for the help he has given throughout its course. I am indebted to Mr T. R. Parsons for supplies of crystalline haemoglobin, and for assistance in the preparation of methaemoglobin.

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XXXVII. IRON DEFICIENCY IN PIGS.

By JOHN POOL McGOWAN and ARTHUR CRICHTON.

From the Rowett Research Institute, Aberdeen.

(*Received December 22nd, 1923.*)

In a previous paper [McGowan and Crichton, 1923] a preliminary account was given of this subject. It is now proposed to give the result of more extended observations.

Having regard to the previous findings, two pregnant sows, during the last few weeks of pregnancy and during lactation, were fed on an iron-poor diet. Their food consisted of distiller's draff, bruised maize and fish meal in the following amounts per day: maize, 2 lbs.; whitefish meal, 1 lb.; distiller's draff as much as they would eat. One sow received in addition 40 g. of ferric oxide per day. This was intimately mixed up with her food. The food was supplied three times a day. They had an abundant water supply. The sows and their pigs were confined during the whole time in concrete pens.

In the case of the sow receiving the iron, the pigs were excluded while the sow was feeding. Thereafter the food was removed. This procedure was carried out to prevent as far as possible the young pigs obtaining iron otherwise than through the milk of the mother. As, however, some of the food was scattered by the sow on the floor of the pen and as, more important, her faeces contained a considerable quantity of iron, the possibility of the pigs obtaining iron in this way could not be entirely excluded.

The young pigs were not allowed outside the pen, were not exercised, and were never separated from their mother except for the short time during which she was feeding.

An attempt was made to estimate the amount of iron in the milks of the two sows. This failed at the outset, owing to inability to obtain enough milk from the sows for this estimation and because of the absence of any reliable method of estimating the very minute quantities of iron present, if small samples of milk were used.

Two sows, Sow 62 and Sow 131, were brought into the pens a fortnight before their farrowing date on April 2 and were made the subject of the experiment already mentioned. The ferric oxide was administered to Sow 131.

They farrowed on April 2, Sow 62 having a litter of eight pigs, numbered consecutively from 1–8, and Sow 131 a litter of ten pigs, numbered from 9–18. As soon as they were born three pigs from Sow 62—pigs 6, 7 and 8—were placed with Sow 131, and four pigs from Sow 131—pigs 15, 16, 17 and 18—were transferred in their places to Sow 62.

Various points in connection with the two groups were observed during the course of the experiment and these are summarised in Tables I and II.

Table I. *"Non-iron" Sow.*

	March			April						May								June				Remarks
	14	20	28	2	6	15	19	24	29	4	7	10	14	17	22	25	31	4	7	12	22	
Sow 62.				Farrowed																		
Haemoglobin %	70	80	80		—	—	—	75	75	—	70	—	—	80	—	—	—	—	—	—	—	Alive and well
Red blood cells in millions per c.mm.	6·5	7·5	6·9		—	—	—	5·3	5·4	—	5·0	—	—	—	—	—	—	—	—	—	—	
Pig 1 (Sow).																						
Weight in kilos.	—	—	—	1·263	Dead	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Dead. Overlaid by sow
Haemoglobin %	—	—	—	70		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Red blood cells in millions per c.mm.	—	—	—	—		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Pig 2 (Boar).																						
Weight in kilos.	—	—	—	1·440	—	—	—	—	—	—	—	—	—	—	—	11·5	—	13·8	15·9	18·1	—	Alive. Typical iron deficiency
Haemoglobin %	—	—	—	60	60	55	50	40	40	35	—	40	40	40	35	40	60	60	70	—	—	
Red blood cells in Millions per c.mm.	—	—	—	—	—	—	3·3	2·8	2·7	3·0	—	—	—	—	—	—	—	—	—	—	—	
Pig 3 (Boar).																						
Weight in kilos.	—	—	—	1·390	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Dead in 59 days.
Haemoglobin %	—	—	—	80	60	40	40	30	40	30	—	40	25	25	40	35	—	—	—	—	—	Typical iron deficiency
Red blood cells in millions per c.mm.	—	—	—	—	—	—	3·0	3·0	2·5	2·5	—	—	—	—	—	—	—	—	—	—	—	
Pig 4 (Sow).																						
Weight in kilos.	—	—	—	1·320	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Alive. Typical iron de ficiency
Haemoglobin %	—	—	—	70	70	40	40	35	—	45	—	40	35	40	50	60	60	70	—	70	—	
Red blood cells in millions per c.mm.	—	—	—	—	—	—	3·0	3·6	—	4·2	—	—	—	—	—	—	—	—	—	—	—	
Pig 5 (Boar).																						
Weight in kilos.	—	—	—	1·430	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Dead. Acute obstruction of intestine. Iron deficiency beginning
Haemoglobin %	—	—	—	60	70	40	Dead	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Red blood cells in millions per c.mm.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Pig 15 (Boar).																						
Weight in kilos.	—	—	—	1·240	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Iron deficiency at first, then about 29th April became a "runt" and so killed
Haemoglobin %	—	—	—	80	70	50	40	40	60	—	60	60	65	Killed	—	—	—	—	—	—	—	
Red blood cells in millions per c.mm.	—	—	—	—	—	—	4·5	3·6	6·8	—	6·5	—	—	—	—	—	—	—	—	—	—	
Pig 16 (Boar).																						
Weight in kilos.	—	—	—	1·330	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Dead. Overlaid by sow
Haemoglobin %	—	—	—	60	Dead	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Red blood cells in millions per c.mm.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Pig 17 (Sow).								Fluid in abdomen, prolapse of anus														
Weight in kilos.	—	—	—	1·310	—	—	—	—		—	—	—	—	—	—	—	—	—	—	—	—	Dead in 38 days.
Haemoglobin %	—	—	—	70	55	45	40	40	40	30	25	30	Dead	—	—	—	—	—	—	—	—	Typical iron deficiency
Red blood cells in millions per c.mm.	—	—	—	—	—	—	4·1	2·7	3·0	2·8	3·0	—	—	—	—	—	—	—	—	—	—	
Pig 18 (Sow).																						
Weight in kilos.	—	—	—	1·290	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Dead in 48 days.
Haemoglobin %	—	—	—	70	60	40	30	30	25	25	20	20	20	20	Dead	—	—	—	—	—	—	Typical iron deficiency
Red blood cells in millions per c.mm.	—	—	—	—	—	—	1·0	2·0	2·0	2·0	1·8	—	—	—	—	—	—	—	—	—	—	

Table II. "*Iron*" *Sow.*

		March			April						May							June			
		14	20	28	2	6	15	19	24	29	4	7	10	14	17	22	25	31	7	12	22	Remarks
Sow 131.	Haemoglobin %	70	80	80	Farrowed	80	80	—	80	75	—	80	—	—	80	—	—	—	—	—	—	Alive and well
	Red blood cells in millions per c.mm.	5·9	7·5	6·0		—	—	—	6·4	5·7	—	6·0	—	—	—	—	—	—	—	—	—	
Pig 6 (Sow).	Weight in kilos.	—	—	—	1·360	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Alive and well.
	Haemoglobin %	—	—	—	70	60	60	60	60	70	70	—	65	75	70	80	70	—	—	—	—	No iron defi-
	Red blood cells in millions per c.mm.	—	—	—		—	4·0	4·4	3·5	5·0	—	—	—	—	—	—	—	—	—	—	+	ciency
Pig 7 (Boar).	Weight in kilos.	—	—	—	1·540	—	—	—	—	—	—	—	—	—	—	—	—	16·3	20·4	22·7	28·4	Alive and well.
	Haemoglobin %	—	—	—	60	70	70	60	70	70	—	—	70	75	75	80	80	—	—	—	—	No iron defi-
	Red blood cells in millions per c.mm.	—	—	—		—	3·7	4·9	5·8	—	—	—	—	—	—	—	—	—	—	—	—	ficiency
Pig 8 (Boar).	Weight in kilos.	—	—	—	1·440	—	—	—	—	—	—	—	—	—	—	—	—	15·9	19·3	21·3	23·4	Alive and well.
	Haemoglobin %	—	—	—	70	60	60	60	60	60	70	—	60	75	70	75	80	—	—	—	—	No iron defi-
	Red blood cells in millions per c.mm.	—	—	—		—	5·7	4·4	5·7	5·5	—	—	—	—	—	—	—	—	—	—	—	ciency
Pig 9 (Boar).	Weight in kilos.	—	—	—	1·240	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Killed on May
	Haemoglobin %	—	—	—	70	60	45	40	45	40	—	40	40	40	40	40	40	—	—	—	—	25th. Slight
	Red blood cells in millions per c.mm.	—	—	—		—	3·7	Diarrhoea, big belly				3·0	—	—	—	—	—	—	—	—	—	iron deficiency
Pig 10 (Sow).	Weight in kilos.	—	—	—	1·450	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Alive and well.
	Haemoglobin %	—	—	—	60	60	70	—	60	65	70	—	80	75	80	70	80	—	—	—	—	No iron defi-
	Red blood cells in millions per c.mm.	—	—	—		—	—	—	4·8	6·8	7·1	—	—	—	—	—	—	—	—	—	—	ciency
Pig 11 (Sow).	Weight in kilos.	—	—	—	1·250	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Alive and well.
	Haemoglobin %	—	—	—	60	70	70	60	80	70	—	—	80	75	80	75	75	—	—	—	—	No iron defi-
	Red blood cells in millions per c.mm.	—	—	—		—	—	5·6	7·0	6·4	—	—	—	—	—	—	—	—	—	—	—	ciency
Pig 12 (Sow).	Weight in kilos.	—	—	—	1·050	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Alive. Slight
	Haemoglobin %	—	—	—	70	70	60	50	60	60	—	—	40	40	60	80	70	—	—	—	—	iron deficiency
	Red blood cells in millions per c.mm.	—	—	—		—	—	4·5	4·1	3·6	—	—	—	—	—	—	—	—	—	—	—	
Pig 13 (Boar).	Weight in kilos.	—	—	—	1·250	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Alive and well.
	Haemoglobin %	—	—	—	70	70	60	65	—	—	60	—	60	70	70	70	80	—	—	—	—	No iron defi-
	Red blood cells in millions per c.mm.	—	—	—		—	—	4·1	—	—	5·0	—	—	—	—	—	—	—	—	—	—	ciency
Pig 14 (Boar).	Weight in kilos.	—	—	—	1·250	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Alive and well.
	Haemoglobin %	—	—	—	70	70	65	—	70	—	65	—	60	65	80	80	75	—	—	—	—	No iron defi-
	Red blood cells in millions per c.mm.	—	—	—		—	—	—	5·0	—	6·0	—	—	—	—	—	—	—	—	—	—	ciency

Before discussing them, the symptoms and pathology of the condition of iron deficiency may be briefly stated: this will facilitate reference in the tables. The disease, although actually commenced some time previously, as will be seen later, usually declares itself when the pigs are about three weeks old. The pigs affected are very pale in colour, very fat and stocky in build and become more and more listless. Often there is blueness of the ears and along the back of the body. Spasmodic breathing develops—"thumps"—and a great many of the animals die quite suddenly at this stage. Diarrhoea is a very prominent feature and great thirst is very often present. The temperature is normal or subnormal. The haemoglobin may be as low as 20 %. On examining such cases, post mortem, the heart is found to be greatly dilated, there is great excess of pericardial fluid, the lungs are oedematous with sometimes effusion into the pleural cavities, and there is great effusion into the peritoneal cavity. The liver is studded all over the surface and in sections with minute white dots, indicative of fatty change in the centre of the lobules. In the tables, the term "typical iron deficiency" means that in such cases all the most important symptoms and signs just enumerated were present.

The occurrence of iron deficiency in the pigs of the two groups may now be presented in the form of a table—Table III.

Table III. *Sow* 62, "*Non-iron*" *Sow.*

Pig	Lived	Died	Cause of death	Iron deficiency present
1	5 days	Yes	Overlaid	...
2	3 months +	No	...	+
3	54 days	Yes	Iron deficiency	+
4	3 months +	No	...	+
5	16 days	Yes	Acute intestinal obstruction	? + beginning
15	46 „	„	Killed as "runt"	+ then recovered
16	5 „	„	Overlaid	...
17	39 „	„	Iron deficiency	+
18	49 „	„	„	+
		Sow 131, "*Iron*" *Sow.*		
6	3 months +	No	No	No
7	„ „	„	„	„
8	„ „	„	„	Trace
9	„ „	„	„	No
10	„ „	„	„	„
11	„ „	„	„	„
12	„ „	„	„	Trace
13	„ „	„	„	No
14	„ „	„	„	„

The great preponderance of cases, and these fatal, of iron deficiency in the "non-iron" group will be observable. Other points in connection with these tables, especially the occurrence of the iron deficiency in the "iron" group will now be discussed.

During the whole time of the experiment the haemoglobin titre and the red blood cell count of the two sows remained within slight limits practically steady. This is especially noteworthy in connection with Sow 62. (See Tables I and II.)

The haemoglobin of the young pigs from both sows estimated[1] at birth with blood obtained from the umbilical vessels, ranged from 60–80 %, and was the same for the pigs of both sows.

With the exception of pigs 9 and 12, which will be specially referred to, all the pigs of Sow 131—the "iron" sow—showed throughout the experiment a constant level of haemoglobin titre from 60–80 % and a red blood cell count ranging from 4–6 millions per c.mm. This was quite distinct from what occurred in the case of the pigs of the "non-iron" sow—Sow 62. Here the haemoglobin titre fell within the first fortnight below 50 and remained there, reaching in some cases as low as 20 %. Apparent exceptions where, after the primary fall, there was a subsequent rise, as took place in the cases of pigs 15, 2 and 4, will be discussed later. Again in this group the red blood cell count was lower than in the "iron" group, being in most cases 3 million per c.mm. or below. (See Tables I and II.)

It will thus be seen from these haemoglobin estimations that the disease was present in the animals for some considerable time prior to the onset of the sudden deaths, which directed attention to its presence. This point is of some importance in connection with cotton seed poisoning, in that in this condition the occurrence of the sudden deaths, in what are alleged to have been the healthiest and best animals, is adduced by some as evidence that cotton seed injury is in reality caused by a poisonous substance acting presumably at the moment of death. In the condition of iron deficiency, in a similar way, the uninitiated consider that the pale, fat, stocky pigs, the subjects of iron deficiency and practically moribund, are in the best of health and much better than the leaner and more active and ruddier pigs which have an abundant iron supply. The sudden death on which importance is laid in both instances is, of course, due to the great dilatation of the heart, and the water-logged condition of the body which are culminating features in a disease which has existed for some time unrecognised.

Reference may now be made to the exceptional cases in both groups. In pig 15, the haemoglobin titre, after a preliminary fall rose again. This rise was associated with a corresponding rise in the red blood cell count, but also and more important, with a stoppage of growth of the animal. It became a runt and on being killed showed none of the acute signs of iron deficiency. In particular, the liver showed no evidence of disease.

This association of the disease with growth of the animal is more particularly brought out in the cases of pigs 2 and 4. Here, during the period 2nd April to the 22nd May, it was particularly noticed that these pigs were growing much faster than their litter mates 7 and 8 on Sow 131. Unfortunately, their weights were not taken. It was then noticed that while pigs 7 and 8 were progressing uniformly, pigs 2 and 4 were at a standstill or even getting less in weight. This stoppage of growth was associated with a rise in the

[1] Examination was made with an instrument standardised for human blood. Normal sow's blood gave with this a reading of from 70 to 80 %.

haemoglobin titre and the assumption of a "runtish" look. The weights of the two lots were now taken (see Tables I and II) and it was found that pigs 7 and 8 were heavier than pigs 2 and 4 and that they were putting on weight at a greater rate. It may be mentioned that pigs 7 and 8 showed no variation in their haemoglobin titre throughout the whole experiment.

With the fact of the association of the disease with the rate of growth before us, we now come to a discussion of the exceptional cases in the "iron" group. These are pigs 10 and 12. At the outset, it may be noted that these are milder cases than those which occurred in the "non-iron" group. Neither of the pigs died, although one indeed was killed when it was about two months old. This was done because the animal had served its purpose in the experiment, and because, being a waster, it was not profitable to keep it. The occurrence of the disease at all in this group has however to be explained. As we know from practical experience of a year's duration in a pig farm attached to the Rowett Institute with about 40 breeding sows, the use of ferric oxide effectively prevents the appearance of the disease. It cannot therefore be, unless the experiment was performed on an exceptional sow, that the administration of the iron to the sow caused a rise in the iron content of the milk. Otherwise, these cases should not have arisen. We have already alluded to the reason why the iron content in the milk of the two sows was not estimated directly. On the other hand, attention, as far as this was practicable, was paid to preventing the pigs from obtaining iron otherwise than through the milk. It has been already mentioned, however, that iron absorption by this means could not be altogether excluded and it is probable that the young pigs of this litter obtained some iron by nibbling the faeces of the mother and the scattered food on the floor of the pen. This would to some extent prevent the disease appearing, but another factor has to be taken into consideration in this case. From experience in the large piggery, it is well recognised that the pigs most liable to the disease are those which are growing fastest, and conversely slow-growing pigs and runts in litters where the rest are dying from the disease escape it. In the present case, Sow 131 suckled throughout the whole experiment 9 pigs, while Sow 62 suckled only 6, which number was soon reduced by deaths from the disease. Again it was very noticeable that the pigs on Sow 62 were growing much faster than those on Sow 131. This was particularly observed in reference to the litter mates 2 and 4 and 7 and 8, the first two on Sow 62 and the last two on Sow 131.

It would appear therefore that the reason for the occurrence of the disease in the pigs of Sow 131 was that the feeding of the iron to the sow did not increase the amount of iron in the milk. On the other hand the reason why the disease did not appear in all her pigs as in the case of Sow 62, and appeared in such a mild degree was that the young pigs on Sow 131 obtained iron by eating the faeces of their mother and were less liable to the disease in that they were growing slower.

These conclusions are confirmed by the precautions taken to prevent the disease in the large piggery already referred to. A year ago, as many as

70 pigs died in a month from the disease. At the present time, while the number of breeding sows has increased and while farrowing is proceeding at a greater rate, it is difficult, owing to the precautions taken, to obtain a case of the disease for demonstration purposes.

The precautions taken are briefly as follows. The sow's ration contains a large quantity of ferric oxide. This ensures a rich source of iron in the pen to which the young pigs have access. The young pigs are encouraged by every means possible to eat of this food. Thus they are separated from the sow for stated periods daily. This prevents them continuously sucking the sow and makes them hungry, in which case they are induced to eat the iron-containing food lying about. Their appetite is further increased by their being given exercise outside in a grass field which gives them an opportunity of obtaining iron by rooting in the soil and eating grass. The exercise further minimises the risk of their becoming too fat which, although a direct consequence of the disease, would appear also to predispose to it.

The disease dealt with here is one of the suckling stage, being either initiated then, or having its most fatal or dangerous period at that time. As the young pigs grow older and their diet becomes less and less milk and more and more extraneous food, the disease is not so liable to occur. Moreover, as this happens, pigs already affected begin to get better. This is illustrated in the cases of pigs 2 and 4 (see Table I) where after being affected acutely, and without alteration of the diet, recovery took place. That the diet, although poor in iron, contained sufficient for their needs, is shown by the fact that the sow herself remained on this diet for the duration of the experiment without any lowering in the titre of her haemoglobin. That milk although an ideal food in every other respect, is a very poor food as regards its iron content need not be stressed.

The ration by which the disease was experimentally produced above is admittedly one poor in iron: but the disease occurs very widely and can be produced on quite a variety of diets, provided other conditions are favourable. An instance may be cited where it occurred on the following diet: fish meal, $1\frac{1}{2}$ lbs.; white sharps, $2\frac{2}{7}$ lbs.; bran, $1\frac{1}{14}$ lbs.; bruised oats, $1\frac{1}{4}$ lbs.; potatoes, 8 lbs.; turnips 10 lbs.; treacle $\frac{1}{3}$ lb. per sow per day. This diet in virtue of the bran contained a fair amount of iron. The sows and pigs however were confined in cement pens and no attention was paid to exercise or encouragement of the young pigs to eat as soon as possible. In this case, too, the young pigs were receiving extra cow's milk; but this had no effect in prevention of the disease as might be expected.

The disease is not of an infectious nature. No bacterial or toxic condition, although both have been carefully sought for, can be assigned as having a causal relation. For the early diagnosis of the disease the estimation of the haemoglobin would appear to be the most reliable method. Early diagnosis is important, for the longer the disease is allowed to go on without appropriate treatment the more difficult it becomes to influence, and the larger will be the percentage of runts and wasters which survive.

In a previous paper [1923] it was mentioned that the disease might have an important bearing on the subject of "wet" beriberi in human beings. The resemblance is very striking, including the suggestive fact that while rice bran contains 0·232 % of iron, polished rice contains only 0·003 %. We know of no proof that "wet" and "dry" beriberi are aetiologically the same and it may quite well be that they are distinct in origin. In the present case, moreover, the disease was produced in pigs, the mothers of which were fed to a very large extent on draff[1], a material, which in consideration of its composition, must contain a fair amount of water-soluble vitamin. The question of the lack of the anti-scorbutic vitamin does not arise here. On the other hand, considering the food supplied and the conditions in which the sow and her pigs were kept, absence of fat-soluble vitamin would seem at first sight to be a likely possibility. The disease produced has, however, nothing in common with the type of disease associated with the lack of this vitamin. The specific curative and preventive effects, moreover, of iron in the shape of ferric oxide have to be considered. In our previous communication, to quote an example of this it was recorded that, on the administration of ferric oxide to animals already affected, the sudden deaths ceased and the symptoms abated, and together with this the haemoglobin titre as estimated on about one hundred animals, before and after the administration of the iron, rose in three weeks from 20–30 % to 70–80 %.

We have previously drawn attention to the resemblance of this disease to cotton seed poisoning [1923], in respect of the symptoms, post mortem appearances, and the beneficial effects resulting from the use of iron. We have made further observations on this subject, but we propose to deal with them in a separate paper.

Summary and Conclusions.

1. A condition in suckling pigs is described and experiments in connection therewith detailed. This condition is attributable to a lack of iron in the sow's milk.

2. It can be readily prevented and cured by the administration of ferric oxide in the food put into the sow's pen. This result in all probability is brought about by the provision in this way of an easily accessible and rich store of iron for the young pigs, which they should be induced by every means possible to partake of as soon and as liberally as possible. In all probability the effect is not produced by an increase of the iron in the milk by the sow being fed on such food.

3. The possible relation of the condition to "wet" beriberi and to avitaminosis in general is discussed.

[1] Draff is malted barley after extraction with water. The material is fed wet and thus contains a considerable amount of the extraction water.

REFERENCE.

McGowan and Crichton (1923). *Biochem. J.* **17**, 204.

XXXVIII. COTTON SEED MEAL POISONING.

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(*Received December 22nd, 1923.*)

For many years a condition of illness of animals attributed to the feeding of cotton seed meal has been observed in America. This has been called by some "poisoning," by others "injury," reflecting thus the somewhat obscure condition of knowledge regarding it.

It is difficult to obtain a clear conception of the disease from the literature. Friedberger and Fröhner [1910] state that only young animals are usually affected. They are said to show disorder of the digestive and urinary apparatus. The duration is variable. The autopsy reveals in the acute form haemorrhagic gastro-enteritis, opaque swelling of the liver and kidneys, oedema of the lungs, dark red urine and collection of liquid in the cavities of the body. There is no enlargement of the spleen. In chronic cases only one finds general emaciation and dropsical phenomena but at times also parenchymatous nephritis. Hutyra and Marek do not discuss the condition in their standard work on animal pathology.

Rommel and Vedder [1915] state that among the more pronounced symptoms observed in pigs suffering from the effects of cotton seed meal feeding are diarrhoea, a harsh rough curly coat, paralysis and shortness of breath. Emaciation and dropsical conditions are frequently observed. The disease manifests itself in two forms, acute and chronic. The acute form is said to be much more serious to the farmer because pigs are attacked by it with little warning before any indications of disease are noticed. The largest and best nourished pigs are often the ones attacked. The attack is sudden and sharp. The pig experiences extreme shortness of breath and suffers the most intense pain. If he recovers, recurrences of the attack are likely especially if the pig is a heavy feeder. Subsequent attacks may end fatally or the disease may assume the chronic form. In the chronic form fatal results may not occur for a considerable time. The symptoms persist if the feed is not changed and the pig appears to develop a certain degree of immunity to the effects of the disease. His condition however is continually although slowly declining. Pigs suffering from this form of disease may live for a year or more on a cotton seed meal ration.

On post mortem examination, pigs which have died from the effects of cotton seed meal feeding show large quantities of fluid in the abdominal and thoracic cavities and in the pericardial sac. The kidneys, liver, spleen, and

small intestines are usually congested. In some cases the membrane lining the stomach is eroded. The lungs are very oedématous especially in pigs which have died from sudden acute attacks. The heart is enlarged.

Wells and Ewing [1916] state that the post mortem results in their cases varied so widely as to be incapable of definite interpretation. Among the characteristics found most constantly are mentioned a rough coat, dry, harsh and scaly skin, weakness and emaciation as shown by physical appearance and entire absence of fat from kidneys and mesentery, and bones usually though not always brittle. Their experiments were performed on six weeks old pigs weighing about 6–10 kilograms at the beginning of the experiment. They state (pp. 18 and 22) that the injury from cotton seed meal in their cases was not acute. Through physical appearances and the metabolic characters it was manifested long before death. This they state is not in agreement with the observations of many other investigators. They are inclined to believe however that in the reported cases of acute cotton seed meal injury, careful periodic measurements would have disclosed the injury before death. This according to them is important: for if well-nourished animals die of the injury as often claimed, the same cannot be due to a deficient diet and inanition but must be due to a toxic effect.

Withers and Carruth [1917] in dealing with the effect of iron as an antidote to cotton seed meal poisoning, give little information as to the symptoms and post mortem appearances of their animals. On p. 256 they state that in one experiment all the pigs were in extremely poor condition at its termination. The gains were slight and the animals were found to be badly infested with internal and external parasites. Post mortem examination showed that practically all the animals were in a pathological condition, the nature of which however is not stated.

In the *Farmer's Bulletin* [1923], which we have just received, cotton seed meal is stated to produce inflammation of the digestive organs and congestion of the lungs in pigs. Fluid is found in the thoracic and the abdominal cavities and the fatty tissues have a yellow cast. We emphasise this last point because it in all probability indicates the presence of jaundice, a condition which we have found in our cases, associated with extensive changes in the liver.

It will thus be seen that the literature dealing with cotton seed meal poisoning in the pig, to which we have confined ourselves, is small in amount and somewhat indefinite in character. The symptoms etc. attributed to cotton seed meal poisoning would appear to divide the alleged cases roughly into two groups. The first group, styled the chronic group, would appear to show nothing characteristic. It is apparently differentiated by the fact that subsequent to the feeding of cotton seed meal, there developed in the pigs inanition, loss of weight and failure to grow. The second group is termed the acute group apparently from the fact that in such cases the animals die suddenly and exhibit post mortem well-defined lesions such as dropsy, oedema of the lungs, enlarged heart, congestion of the liver, etc.

From our survey of the literature it does not appear that the possibility of other diseases occurring in the experimental pigs either prior to or synchronous with the experiment have been sufficiently taken into account in considering the results obtained by feeding pigs on cotton seed meal. Again the view that sudden death in apparently well nourished pigs which have been fed on cotton seed meal is to be interpreted as a toxic action would appear not to be justifiable. It is not logically sound and as will be seen later is in all probability not correct in this particular instance. For the present it may be mentioned that this sudden death is admitted by those making this interpretation to have been associated with great enlargement of the heart and dropsy throughout the body, conditions which must have existed for some time prior to death.

On February 17, 1923, an experiment was begun with the object of reproducing if possible the injurious effects of cotton seed meal and to test the effect of iron in the form of ferric oxide in preventing them. Two lots of pigs, aged about nine weeks old, were put into concrete pens—pens 1 and 2. The pigs in pen 1 were fed on a mixture of cotton seed meal and maize in the following proportions, cotton seed meal 4 lbs., and maize, 12 lbs. Those in pen 2 were fed on the same ration with in addition 40 g. of ferric oxide to the above quantity of ration. Of these rations which were fed three times a day the pigs were allowed to eat as much as they liked. The pigs in pen 1 were numbered as follows, 654, 540, 648 and 631 and those in pen 2, 655, 548, 646 and 629.

The results of this experiment are detailed in Tables I and II. The experiment lasted about three months, from 17th February, 1923, to May 12, 1923. From the tables it will be seen that the pigs in pen 1, after an initial slight rise in weight, thereafter steadily fell off. Thus on April 3rd, after about seven weeks, the weights of all of them were much lower than their initial weights on commencement of the experiment. By this time, it should be noted, they had almost given up eating their ration. On April 6th, while the ration was continued, they were given in addition a mixture of salts, with a composition as follows: bone meal 3 ounces, chalk 1·5 ounces per ration as above. The mixture contained no iron. This effected very little improvement in their condition although for a short time thereafter they ate their ration better. The haemoglobin of the various animals in this group was estimated at frequent intervals and showed throughout an absence of any haemoglobin deficiency.

The pigs in this group were killed on the various dates indicated in the table. Post mortem nothing was found except extreme emaciation.

The pigs in pen 2 as a whole were slightly larger pigs to begin with than those in pen 1. The experiment with them lasted over the same period. In all of them the weight on April 3rd (seven weeks) exceeded that at the beginning of the experiment, although in the interval the weights had been somewhat irregular, never however falling below the initial weight. During the whole time this group showed an avidity for its ration. On April 6th the

ration being continued they were given in addition the same salt mixture as pen 1. In all of them this caused a slight fillip to the putting on of weight. The experiment was terminated on May 12th, all animals being alive in fair condition and all being considerably heavier than they were at the commencement of the experiment.

During the whole course of the experiment there was no evidence of haemoglobin deficiency.

The pigs in pen 1 therefore, fed on cotton seed meal and maize without iron, in a short time, without diminution of their haemoglobin titre, ceased to grow and eventually were reduced to a state of great emaciation. Associated with this was the fact that the pigs refused to eat the food. How far this was due to the ration being unpalatable it is difficult to say. The food mixture had as fed an undoubtedly peculiar odour. The pigs in pen 2, however, fed on the same ration with the addition of ferric oxide, ate their ration well and put on weight all the time. There was no haemoglobin deficiency. Withers and Carruth [1917] found that iron sulphate lessened the detrimental effects of cotton seed meal, a point which will be discussed later. It will be seen thus that typical cotton seed meal poisoning appeared in neither of our two groups, the non-iron or the iron group. The probable reason for this will be discussed later.

While the above experiment was in progress, three other groups of pigs in adjacent pens were on feeding experiments which eventually turned out to have a considerable bearing on the present problem as certain of them developed the symptoms and post mortem appearances of the acute form of cotton seed meal poisoning. The first group consisted of four pigs, numbered 656, 545, 645 and 627, about nine weeks old which were housed in pen 3. Their ration consisted of maize 12 lbs. and earth nut meal 4 lbs. With this they were fed three times a day and received as much as they could eat of the mixture. The results connected with this group are summarised in Table III.

The experiment commenced on February 17th, 1923, and lasted for approximately three months. In all the animals there was a fall in weight after a preliminary rise, the final weight in two of them (656 and 545) being however considerably higher than the initial weight. The haemoglobin titre remained normal in pig 656, but in the other three it fell considerably to 45 % in pig 545, 30 % in pig 645, and 40 % in pig 627. In addition these three pigs showed blueness of the ears and along the back, conditions indicative of considerable circulatory weakness. Their temperature was not raised but there was considerable thirst. All three showed spasmodic jerky breathing—"thumps." Pig 545 died suddenly on April 1st and pig 645 suddenly on April 3rd. Both pigs were in fairly good condition. Post mortem there was found in both great effusion of fluid into the serous cavities, great enlargement and marked congestion of the liver, together with the other changes in that organ already described in iron deficiency in sucklings, such as fatty change

Table I. *Haemoglobin estimated with an instrument standardised for human blood on which* 80 % = 100 % *pig's blood.*

Pen 1 Pig	1923	February			March						April						May			
		17	22	27	4	9	14	19	24	29	3	8	12	16	23	28	3	8	12	
654	Weight in lbs.	37·4	14·7	36·1	36·3	38·3	38·5	36·3	34·3	30·5	30·3	32·5	30	29	30·5	31	31	32	29	Killed, emaciated,
	Haemoglobin %	—	—	—	80	70	70	—	70	80	80	70	—	80	60	70	70	—	70	no disease
540	Weight in lbs.	40	40	41·3	40·9	41·8	40·3	38·9	39·4	38	35	34·5	Killed, emaciated, no disease							
	Haemoglobin %	—	—	—	70	70	60	—	75	65	75	75								
648	Weight in lbs.	35·6	36·7	38·5	38·1	37·4	35·6	37·2	34·5	32	32	—	Killed, emaciated, no disease							
	Haemoglobin %	—	—	—	80	70	70	—	70	60	70	70								
631	Weight in lbs.	48·4	49·5	50·4	50·4	51·3	47·7	44	47·3	43	41·7	41	39	39	41·5	39·5	405·	40	39·5	Killed, emaciated,
	Haemoglobin %	—	—	—	80	70	70	—	75	70	80	70	—	80	60	70	80	—	80	no disease
Pen 2																				
655	Weight in lbs.	44·4	45·1	49·9	49·5	51·3	52·8	54·3	55·7	55	52	53	52	49·5	52	50·5	55	58	60·75	Alive and well, no
	Haemoglobin %	—	—	—	80	75	80	80	—	60	70	80	—	70	70	65	70	—	80	signs of disease
548	Weight in lbs.	49·5	52·8	58·1	58·5	60·5	60·3	63·1	64	65·7	65·5	67·5	71	71·5	76·5	75	77	79·5	81·5	,, ,,
	Haemoglobin %	—	—	—	80	75	80	75	—	80	75	80	—	70	75	80	70	—	70	
646	Weight in lbs.	46·8	48·8	54·5	54·3	51·9	53	50·2	49·7	48·5	50	50·5	50·5	50·2	55·5	52·5	56	56·5	56·5	,, ,,
	Haemoglobin %	—	—	—	75	80	75	75	—	80	70	75	—	75	80	75	80	—	80	
629	Weight in lbs.	44·4	44·9	49·5	52·8	49·7	52·6	53·7	51·5	52	52	52	53	52	57	55	56	56	51	,, ,,
	Haemoglobin %	—	—	—	80	70	75	—	80	80	75	60	—	70	70	80	80	—	80	

Table II. *Haemoglobin estimated with an instrument standardised for human blood on which* 80 % = 100 % *pig's blood.*

Pen 3 Pig	1923	February			March						April						May			
		17	22	27	4	9	14	19	24	29	3	8	12	16	23	28	3	8	12	
656	Weight in lbs.	42·9	43·1	45·3	46·2	48·2	51	49·3	49·5	47·2	43	48	Put on to ordinary diet with iron in addition							
	Haemoglobin %	—	—	—	80	70	80	—	80	70	75	—	—	—	80	—	70	—	—	
545	Weight in lbs.	52·6	57·4	65·1	68·4	73	75·9	74·4	72·8	64·5	—	—	—	—	—	—	—	—	—	
								Very blue ears, dead												
	Haemoglobin %	—	—	—	80	80	75	60	60	45	—	—	—	—	—	—	—	—	—	
645	Weight in lbs.	47·9	48·8	53·7	51	51	51·3	46·6	46	42	—	—	—	—	—	—	—	—	—	
								Very blue ears, dead												
	Haemoglobin %	—	—	—	80	70	60	60	40	30	—	—	—	—	—	—	—	—	—	
627	Weight in lbs.	41·6	43·1	46·4	46·4	47·3	48·8	44·2	42·3	36	34·5	36·5	Put on to ordinary diet with iron in addition							
								Very blue ears												
	Haemoglobin %	—	—	—	80	75	70	—	40	45	60	—	—	—	60	—	70	—	—	
													Pig recovered							

On 26th April ferric oxide in large quantity was added to the ration.

in the centre of the lobules, etc. Pig 545 showed considerable jaundice. Great enlargement of the heart and oedema of the lungs were present in both.

Pig 627, which was badly affected, and pig 656 had ferric oxide added to their ration on April 26th. They were placed on ordinary diet with a continuation of the ferric oxide on May 8th. Subsequent to this they put on weight and were eventually marketed in good condition. The addition of the iron to the diet is reflected in the haemoglobin titre of pig 627 on April 3rd.

In this group fed on maize and earth nut meal two pigs and probably a third developed the appearances described as typical of acute cotton seed meal poisoning. Rommel and Vedder [1915] describe the production of a similar condition in pigs by feeding them on steamed polished rice and tankage. Moore [1914] and Hadwen [1915] describe like occurrences.

The other feeding experiment with pigs which has a bearing on the present condition is as follows. Two groups of four pigs each of about nine weeks old were fed on a mixture of middlings, locust bean meal, palm kernel meal, earth nut meal, and treacle. The proportions of the various constituents present is not known as the mixture was a proprietary one. One lot of pigs received in addition a mixture of salts to bring the food up approximately to the mineral composition of cow's milk, with iron in slight excess. The chemical constitution of the two rations as fed to the pigs was estimated by Mr Godden, to whom we are indebted for the analysis. It was as follows:

	CaO %	P_2O_5 %	Cl %	Fe_2O_3 %	N %	Fat %	Fibre %	Dry matter %
Without salts	0·52	1·84	0·07	?	3·20	5·1	7·65	86·25
With salts	1·54	1·67	0·48	0·05	3·08	5·1	7·65	87·85

Table III. *Haemoglobin estimated with an instrument standardised for human blood on which* 80 % = 100 % *pig's blood.*

		1923 February		March						April	
Pen 9 Pig		17	27	4	9	14	19	24	29	3	8
615	Weight in lbs.	41·1	46·4	44·2	45·3	51·9	53·9	55·6	59	59	63
	Haemoglobin %	—	—	—	—	—	—	—	—	80	—
652	Weight in lbs.	34·5	36·1	37·2	40	45·1	47·7	50·8	55	57·5	63
	Haemoglobin %	—	—	—	—	—	—	—	—	70	—
636	Weight in lbs.	37·4	43·3	44·4	47·1	53·7	55	61·2	63	63	69
	Haemoglobin %	—	—	—	—	—	—	—	—	80	—
541	Weight in lbs.	42·6	47·9	48·5	52·8	59·6	60·3	66·4	69	73	80
	Haemoglobin %	—	—	—	—	—	—	—	—	70	—
Pen 10											
651	Weight in lbs.	32·1	35·8	36·5	38	40·9	41·8	42·9	41	41	40
	Haemoglobin %	—	—	—	—	—	—	—	—	70	—
613	Weight in lbs.	35·6	39·8	40·7	53·8	45·5	44·6	39·2	36	—	Dead on 4. iv. 23
	Haemoglobin %	—	—	—	—	—	—	—	—	50	
638	Weight in lbs.	44	55·9	55·6	60·5	65·4	64·4	65·6	67	64	63
	Haemoglobin %	—	—	—	—	—	—	—	—	80	—
544	Weight in lbs.	43·5	48·4	50·6	52·8	56·3	55·6	54·3 Blue ears	57	52	Dead on 5. iv. 23
	Haemoglobin %	—	—	—	—	—	—	—	—	40	—

The pigs were in two lots. Pen 9 contained pigs 615, 652, 636 and 541 and these were fed on the salt-containing food. Pen 10 contained pigs 651,

613, 638 and 544 to which was given the non-salt food. They were fed three times a day and allowed to eat as much as they wanted. The results of this experiment are detailed in Table III.

The pigs in pen 9, on the salt-containing food, grew fairly well and the growth was continuous. The increase however fell far short of what one expects with a good pig food. The haemoglobin titre in all estimated on April 3rd was normal. None of the animals in this group ever showed any signs of illness. Those in pen 10 on the non-salt-containing food on the other hand showed at first an increase of weight but thereafter a recession. The haemoglobin was estimated on April 3rd and gave the following result: pig 651, 70 %; 613, 50 %; 638, 80 %; 544, 40 %. At this time pigs 613 and 544 were obviously ill with blue ears and thumping jerky respiration. Pig 613 in addition showed jaundice. The temperatures were not raised. There was great thirst. Both died suddenly, pig 613 on April 4th and pig 544 on April 5th. Post mortem examination showed exactly similar conditions to those found in pigs 545 and 645 in pen 3. Pig 544 however did not show jaundice. Attention may also be drawn to the fact that it took practically the same time for the animals in this group to die as it did for those in pen 3. The occurrence therefore of cases showing all the symptoms of acute cotton seed meal poisoning in pen 10 on this diet should be specially noted as also its non-occurrence in pen 9 where the same food was given with the addition of salts, including a fair amount of ferric oxide.

In discussing the results obtained it may be mentioned at the outset that the symptoms and post mortem appearances here described as occurring are those we have discussed elsewhere [McGowan and Crichton, 1923, 1924] in connection with iron deficiency in suckling pigs. The only difference is that on the present occasion they are of a more advanced type, especially those found in the liver. In the condition of suckling iron deficiency we showed that the administration of ferric oxide not only restored the haemoglobin titre in a short time to normal but also that it could clear up in time the extensive organic changes present in the liver. It is known also that this disease attacks especially the fastest growing pigs and that the weaklings of a litter may escape while the faster growing litter mates are affected. The development in a pig with the disease of "runtish" characters at once stops its progress. In this connection we instance pig 15, Table I, of our previous paper [1924]. In particular it may be mentioned that this animal being killed a short time after it had become a runt, showed no signs of disease of the liver, the fatty change in the centre of the lobules, although almost certainly present before, having disappeared in the interval.

Before the introduction of the iron medication as a routine into the piggery it was found that a certain number of the pigs, which had had the disease, on being fed on an ordinary generous diet, but not one containing iron especially, lived. Some of them in the process became runts, others of them however grew well and became eventually quite good pigs. In both instances,

the livers on being examined later were found to be healthy or in the intermediate stage of returning to normality, the "fairy-ring" condition already described [1923, p. 206]. Experimental instances of this same occurrence are pigs 2 and 4, Table I, of our previous communication [1924].

It is evident therefore that suckling iron deficiency where it does not kill (leaving out of account the deficiency of haemoglobin and the after effects of the dropsy) leaves behind it on weaning more or less organic affection of the liver. This will be more particularly described in a succeeding paper on the pathology of the condition. It depends on the way the animal is fed thereafter whether this liver condition improves to become normal or progresses to become worse.

We have already emphasised the point that iron deficiency is a disease of the suckling pig. We do not think that the disease will arise for the first time in a weaned pig when fed on an ordinary mixed diet through deficiency of iron in that diet. In our opinion the nature of the diet fed at that time markedly affects the recovery or otherwise from lesions remaining from a pre-existent iron deficiency in the suckling stage.

The experimental results will now be dealt with in the light of these considerations. The pigs used in the five groups were obtained from the piggery at a time, when, although the beneficial effects of iron were recognised, the exact method of its action was not clearly understood. In particular it was thought that the iron might act by increasing the amount of this mineral in the sow's milk, whereas later experience showed that the result was obtained by the young pigs eating the iron themselves and that the aim, in relation to preventing the disease, should be to get the young pigs to eat of themselves as soon as possible. Under the circumstances therefore and considering the difficulties, which we have met with later, of preventing the occurrence of the disease unless the utmost care is taken to get the young pigs to eat the iron-rich food, it is almost certain that the batch of pigs used in this experiment were suffering more or less from slight sequelae of iron deficiency. They did not show it outwardly but in all probability their livers, judging from our other experience, were deranged in greater or lesser degree. Had they been put on to a full generous diet or one containing a large amount of ferric oxide in all probability they would have gone on all right.

In the light of these considerations, the explanation of the experimental results would appear easy. The pigs in pen 1, on the cotton seed meal and maize, ceased to eat, and passed into a state of complete inanition without any of the signs or post mortem appearances of acute cotton seed meal poisoning. Any traces of iron deficiency as sucklings would disappear in the process, as happened in the case of the runts above referred to. The pigs in pens 2 and 9, on a poor ration with abundant iron in one case and iron and salts in the other exhibited the restorative power of iron in removing traces of iron deficiency. At the same time the pigs grew moderately. The pigs of pens 3 and 10, exhibited the effect of a poor ration without iron, in allowing to progress, or possibly furthering the progress of, the sequelae of the pre-

existent iron deficiency, especially as affecting the liver. That the rations were poor ones is shown by the small increase in weight in a period of three months, even in those cases where no disease symptoms appeared.

It will be seen from the above results and from the work of Moore, Hadwen, Rommel and Vedder, that the so-called cotton seed meal posioning symptoms and post mortem appearances are not limited to pigs which have been fed on cotton seed meal. The facts that cotton seed meal injury is said to affect principally young pigs (Friedberger and Fröhner) and that suckling iron deficiency is a very widespread disease are two additional points in favour of the view we have expressed regarding the nature of the cotton seed meal condition.

Withers and Carruth [1918] conclude from their experiments that cotton seed meal injury of swine is due not to deficient diets but to the presence of a toxic substance. In their opinion the toxic substance in cotton seed meal is the derivative of gossypol which they have called *d*-gossypol. Our experiments as just seen do not lend support to this view. These writers recognised the beneficial effect of iron in the condition but attributed the advantages derived from its use to the iron neutralising the toxic substance in the cotton seed meal.

Several writers such as Rommel and Vedder [1915], Richardson and Green [1916, 1917], Osborne and Mendel [1917] and Wells and Ewing [1916] have suggested that the condition is one of dietary deficiency. Of these Richardson and Green, and Osborne and Mendel have worked with white rats so that their results are not directly applicable. Richardson and Green suggest a mineral deficiency as in some degree responsible. Wells and Ewing working with young pigs concluded that cotton seed meal was an incomplete food. This was true even when it was fed with sugar and starch to a wide nutritive ratio. Pigs upon an absolute maintenance diet ate in addition only small quantities of cotton seed meal and were not greatly injured by it. So-called cotton seed meal injury in their opinion was due in large part to inadequate diets.

Rommel and Vedder [1915] advanced the hypothesis that cotton seed meal injury is similar to if not identical with beri-beri in man. They suggested that it was due to a deficiency in water-soluble vitamin. We ourselves suggested the resemblance between suckling iron deficiency and "wet" beri-beri in human beings. As already mentioned we believe the pig condition to be due to deficiency in iron. The fact that the pigs from sows fed largely on distillers' draff[1], which in the quantities fed must have supplied a fair amount of water-soluble vitamin, markedly succumbed to the disease, would seem to indicate, apart from the therapeutic power of iron, that the condition is not one of lack of water-soluble vitamin. The production of the disease in animals fed on peanuts also points in the same direction.

[1] Draff is malted barley after extraction with water. The material as it is fed contains a considerable amount of the extraction water.

Conclusions.

1. Cotton seed meal injury or poisoning in pigs in all probability is not due to a toxic substance in the cotton seed meal.

2. The same condition can be brought about by feeding pigs on other substances concerning which there is no suspicion of toxicity.

3. In all probability cotton seed injury is brought about by feeding young pigs, which have already suffered as sucklings to a greater or less degree from iron deficiency, on an incomplete diet.

4. The incompleteness of the diet would appear to be a general one and not one having reference to absence of vitamins.

5. Iron in the shape of ferric oxide would appear to have a specially beneficial effect in preventing symptoms from arising.

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